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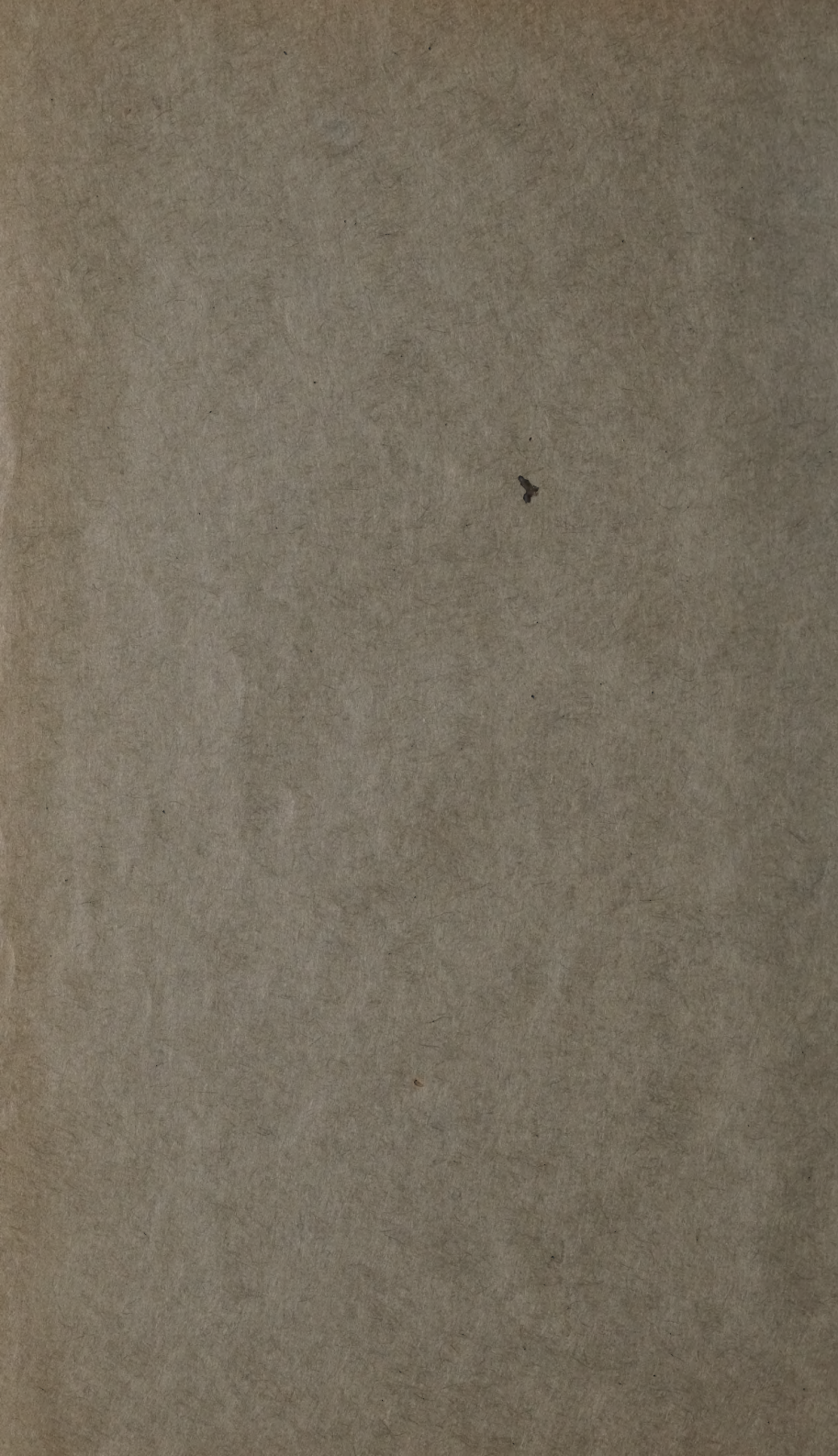
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CONTENTS OF VOLUME LXV.

No. 1, AUGUST, 1925.

	PAGE
MORGULIS, SERGIUS, and BARKUS, O. Studies on glycolysis <i>in vitro</i>	1
DEUEL, H. J., JR., and CHAMBERS, W. H. The rate of elimination of ingested sugars in phlorhizin diabetes.....	7
CHAMBERS, W. H., and DEUEL, H. J., JR. Animal calorimetry. Thirtieth paper. The metabolism of glycerol in phlorhizin diabetes.....	21
LEVENE, P. A., and SIMMS, H. S. Lactone formation from mono- and dicarboxylic sugar acids.....	31
LEVENE, P. A., and HALLER, H. L. The configurational relationships between β -hydroxy acids and α -hydroxy acids and between the latter and secondary alcohols.....	49
RABINOWITCH, I. M. Blood sugar time curves following the ingestion of dihydroxyacetone.....	55
JONES, D. BREESE, MOELLER, OTTO, and GERSDORFF, CHARLES E. F. The nitrogen distribution and percentages of some amino acids in the muscle of the shrimp, <i>Peneus setiferus</i> (L.).....	59
HART, E. B., STEENBOCK, H., ELVEHJEM, C. A., and WADDELL, J. Iron in nutrition. I. Nutritional anemia on whole milk diets and the utilization of inorganic iron in hemoglobin building.....	67
VICKERY, HUBERT BRADFORD. Some nitrogenous constituents of the juice of the alfalfa plant. IV. The betaine fraction.....	81
VICKERY, HUBERT BRADFORD, and VINSON, CARL G. Some nitrogenous constituents of the juice of the alfalfa plant. V. The basic lead acetate precipitate.....	91
McCOLLUM, E. V., SIMMONDS, NINA, BECKER, J. ERNESTINE, and SHIPLEY, P. G. Studies on experimental rickets. XXVI. A diet composed principally of purified foodstuffs for use with the "line test" for vitamin D studies.....	97
HAMILTON, BENGT. A comparison of the concentrations of inorganic substances in serum and spinal fluid.....	101
HJORT, A. M., ROBISON, S. C., and TENDICK, F. H. An extract obtained from the external bovine parathyroid glands capable of inducing hypercalcemia in normal and thyreoparathyroprivic dogs.....	117
ROTHWELL, CARMEN S. Direct precipitation of calcium in cows' milk.....	129
COOK, DONALD H. Temperature coefficients of enzymic activity and the heat destruction of pancreatic and malt amylases.....	135

610683

	PAGE
ANDREWS, JAMES C. The optical activity of cystine.....	147
ANDREWS, JAMES C. The oxidation of cystine.....	161
OKEY, RUTH, and ROBB, ELDA I. Studies of the metabolism of women. I. Variations in the fasting blood sugar level and in sugar tolerance in relation to the menstrual cycle.....	165
LEWIS, HOWARD B. The metabolism of sulfur. IX. The effect of repeated administration of small amounts of cystine.....	187
HENDRIX, BRYON M., and CALVIN, DEB B. The loss of bases in diuresis and its effect upon the alkali reserve of the blood.....	197
WELO, LARS A., and BAUDISCH, OSKAR. On the catalytically active and inactive forms of ferric oxide.....	215
BURRELL, ROBIN C., and PHILLIPS, THOMAS G. The determination of nitrate nitrogen in plants.....	229
WESSON, LAURENCE G. On a possible relationship of arachidonic acid to the saturated fatty acids in fatty acid metabolism.....	235
HOFFMAN, WALTER F. Sulfur in proteins. II. The effect of mild alkaline hydrolysis upon hair.....	251
HELLER, V. G., McELROY, C. H., and GARLOCK, BERTHA. The effect of the bacterial flora on the biological test for vitamin B.....	255
MURRAY, CECIL D., and HASTINGS, A. BAIRD. The maintenance of carbonic acid equilibrium in the body, with especial reference to the influence of respiration and kidney function on CO_2 , H^+ , HCO_3^- , CO_3^{2-} concentrations in plasma.....	265

No. 2, SEPTEMBER, 1925.

DILL, D. B., and ALSBERG, C. L. Preparation, solubility, and specific rotation of wheat gliadin.....	279
LUNDGAARD, CHRISTEN, and HOLBØLL, SVEND AAGE. Studies in carbohydrate metabolism. II. Investigations into the muta- rotation of β -glucose under various conditions.....	305
LUNDGAARD, CHRISTEN, and HOLBØLL, SVEND AAGE. Studies in carbohydrate metabolism. III. Investigations into the nature of the glucose in the blood of normal individuals.....	323
LUNDGAARD, CHRISTEN, and HOLBØLL, SVEND AAGE. Studies in carbohydrate metabolism. IV. Investigations into the nature of the glucose in the blood of patients with diabetes mellitus and of patients with benign glycosuria.....	343
LUNDGAARD, CHRISTEN, and HOLBØLL, SVEND AAGE. Studies in carbohydrate metabolism. V. Investigations into the form of glucose in different body fluids.....	363
HOFFMAN, WALTER F., and GORTNER, ROSS AIKEN. The electro- dialysis of agar. A method for the preparation of the free agar- acid.....	371
HASTINGS, A. BAIRD, SENDROY, JULIUS, JR., and ROBSON, WILLIAM. Studies of acidosis. XXI. The colorimetric determination of the pH of urine.....	381

Contents

v

	PAGE
SUMNER, JAMES B. A more specific reagent for the determination of sugar in urine.....	393
CORI, CARL F., and CORI, GERTY T. The carbohydrate metabolism of tumors. II. Changes in the sugar, lactic acid, and CO ₂ -combining power of blood passing through a tumor.....	397
HENDERSON, L. J., and MURRAY, C. D. Blood as a physicochemical system. III. Deductions concerning the capillary exchange....	407
MURRAY, CECIL D., and MORGAN, WILLIAM O. P. Oxygen exchange, blood, and the circulation. A coordinated treatment of the factors involved in oxygen supply on the basis of the diffusion theory...	419
HASTINGS, A. BAIRD, and SENDROY, JULIUS, JR. The effect of variation in ionic strength on the apparent first and second dissociation constants of carbonic acid.....	445
SALMON, W. D. Vitamin B in the excreta of rats on a diet low in this factor.....	457
LEVENE, P. A., and SOBOTKA, HARRY. Synthetic nucleosides. I. Theophylline pentosides.....	463
LEVENE, P. A., and SOBOTKA, HARRY. Synthetic nucleosides. II. Substituted uracil xylosides.....	469
HADEN, RUSSELL L., and ORR, THOMAS G. Chemical findings in the blood of the normal dog.....	479
LEVENÉ, P. A., and VAN DER HOEVEN, B. J. C. The concentration of vitamin B. II.....	483
JACOBS, WALTER A., and COLLINS, ARNOLD M. Strophanthin. VIII. The carbonyl group of strophanthidin.....	491
LEVENE, P. A., and MIKESKA, L. A. Substitution by halogen of the hydroxyl in secondary alcohols.....	507
LEVENE, P. A., and MIKESKA, L. A. On the oxidation of secondary mercaptans into corresponding sulfonic acids.....	515
LEVENE, P. A., and SIMMS, H. S. The dissociation constants of plant nucleotides and nucleosides and their relation to nucleic acid structure.....	519
LEVENE, P. A., and MEYER, G. M. The numerical values of the optical rotation of methylated gluconic acids and of their salts.....	535
LEVENE, P. A., and ROLF, IDA P. Bromolecithins. I. Fractionation of brominated soy bean lecithins.....	545
LEVENE, P. A., and SOBOTKA, HARRY. The thio-sugar from yeast...	551

No. 3, OCTOBER, 1925.

HARNED, BEN K. The sugar content of blood.....	555
DENIS, W., and LECHE, STELLA. A method for the determination of total sulfates in tissues.....	561
DENIS, W., and LECHE, STELLA. On the distribution of injected sulfates in tissues.....	565
HART, E. B., STEENBOCK, H., and LEPKOVSKY, S. Is the antirachitic factor of cod liver oil, when mixed with ground grains, destroyed through storage? Plate 1.....	571

	PAGE
HART, E. B., STEENBOCK, H., LEPKOVSKY, S., KLETZIEN, S. W. F., HALPIN, J. G., and JOHNSON, O. N. The nutritional requirement of the chicken. V. The influence of ultra-violet light on the production, hatchability, and fertility of the egg.....	579
CLARK, GUY W. Acid- and base-forming elements in foods.....	597
SCOTT, D. A. A further investigation of the chemical properties of insulin.....	601
RABINOWITCH, I. M. Urea tests of renal efficiency. I.....	617
HUNTER, GEORGE, and EAGLES, BLYTHE A. The isolation from blood of a hitherto unknown substance, and its bearing on present methods for the estimation of uric acid. Plate 2.....	623
MAYNARD, L. A., GOLDBERG, S. A., and MILLER, R. C. The influence of sunlight on bone development in swine.....	643
VICKERY, HUBERT BRADFORD. Some nitrogenous constituents of the juice of the alfalfa plant. VI. Asparagine and amino acids in alfalfa.....	657
SMITH, H. MONMOUTH, and DOOLITTLE, DORTHA BAILEY. Energy expenditure of women during horizontal walking at different speeds.....	665
STERN, HANS T. The colorimetric pH test of water or unbuffered solutions.....	677
LEVENE, P. A. The mucoproteins of the snails, <i>Helix aspersa</i> and <i>Helix pomatia</i>	683
VAN SLYKE, DONALD D., HASTINGS, A. BAIRD, MURRAY, CECIL D., and SENDROY, JULIUS, JR. Studies of gas and electrolyte equilibria in blood. VIII. The distribution of hydrogen, chloride, and bicarbonate ions in oxygenated and reduced blood.....	701
ATCHLEY, DANA W., and NICHOLS, EMILY G. The influence of protein concentration on the conductivity of human serum.....	729
STADIE, WILLIAM C., and ROSS, EFFIE C. A micro method for the determination of base in blood and serum and other biological materials.....	735
WILSON, D. WRIGHT, LONG, W. L., THOMPSON, H. C., and THURLOW, SYLVA. Changes in the composition of the urine after muscular exercise.....	755
LILJESTRAND, S. H., and WILSON, D. WRIGHT. The excretion of lactic acid in the urine after muscular exercise.....	773
HJORT, AXEL M. The influence of orally administered calcium salts on the serum calcium of normal and thyreoparathyroprivic dogs.	783

STUDIES ON GLYCOLYSIS IN VITRO.

By SERGIUS MORGULIS AND O. BARKUS.

(From the Department of Biochemistry, University of Nebraska College of Medicine, Omaha.)

(Received for publication, May 18, 1925.)

With the discovery of insulin and its effects on the blood sugar the problem of glycolysis has assumed new and interesting aspects. Is hypoglucemia produced by insulin injections similar to the disappearance of sugar from blood in the *in vitro* glycolytic process? Briggs, Koechig, Doisy, and Weber found that following an insulin injection there is a fall in the blood sugar accompanied by a simultaneous rise in the lactic acid concentration and a lowering of the inorganic phosphates of the blood. This observation would suggest that the mechanism of the *in vivo* glycolysis may possibly depend upon the preliminary formation of a hexose-phosphoric acid compound. Best and Ridout, however, reported recently that the lactic acid of normal and diabetic dogs does not increase in any significant manner during insulin hypoglucemia, unless the latter is also accompanied by extreme asthenia or marked hyperirritability of the animal. This corroborates the earlier observations of Tolstoi, Loebel, Levine, and Richardson who found that in diabetic patients under insulin treatment the lactic acid concentration of the blood generally remains unchanged in spite of the extreme fall in the blood sugar. The inorganic phosphates of the blood, however, do diminish. Servantie likewise fails to observe any relation between the changes in the blood sugar of dogs treated with insulin and the lactic acid concentration of the blood. The discrepancy in the results of several experimenters may possibly be accounted for on technical grounds, when it is recalled that the blood lactic acid is subject to sudden and extensive fluctuations. Thus, Mendel, Engel, and Goldscheider have demonstrated how very sensitive the blood lactic acid is to manipulation of the animal, even slight pressure

at the time of drawing the blood sample causing a considerable increase in the lactic acid content. The study of the influence of insulin is still further complicated by the circumstance that, as Collazo and Supniewski point out, preparations made by different methods give various results as far as the effect on the blood lactic acid is concerned. Some insulin preparations cause a rise while those purified by picric acid have no effect at all on the blood lactic acid. It is also of interest in this connection to mention that Mendel, Engel, and Goldscheider observed that the usual rise and fall in the blood sugar curve following the ingestion of 100 gm. of glucose occurs independently of any alterations in the blood lactic acid, the concentration of which generally remains constant. The weight of experimental evidence seems, therefore, to indicate that the insulin hypoglycemia is not necessarily associated with a production of lactic acid.

Our own experiments deal with the *in vitro* glycolysis. We studied the bloods of several species (horse, sheep, dog, bird) from the point of view of the simultaneous changes taking place in the glucose, lactic acid, and phosphate contents of the blood. The blood was collected aseptically, and a sample was immediately analyzed. The rest of the material was transferred aseptically to a number of test-tubes, stoppered, and left for various lengths of time in a constant temperature incubator.

Analytical Procedures.

The blood sample was deproteinized according to the Folin-Wu procedure and the glucose was determined in an aliquot of the filtrate by the Shaffer-Hartman micro method.

The lactic acid we determined by a modification of Harrop's method.

20 to 30 cc. of the filtrate (or more, if the material was available) were used for each determination. To this was added with constant shaking dry, finely powdered CuSO_4 in sufficient amount to make the final concentration 15 per cent. After the added CuSO_4 had completely dissolved finely powdered $\text{Ca}(\text{OH})_2$ was added, enough to give a 10 per cent suspension of the hydroxide, and the mixture was shaken vigorously for 15 minutes. The mixture was then centrifuged and the supernatant liquid decanted through a small filter paper. An aliquot of this perfectly clear filtrate was now used for the lactic acid determination. By adding the reagents in the dry state it was possible to keep the final volume down, a

TABLE I.

Duration of glycolysis.	Blood.			Remarks.
	Glucose.	Lactic acid.	Inorganic P.	
Oxalated sheep blood.				
hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
0	70	26.6	2.5	No hemolysis.
2	70	26.0	2.5	" "
4	42	33.0	2.5	" "
6	31	37.0	2.5	" "
10	18	41.0	2.5	" "
24	18	42.0	2.5	" "
48	13	58.0	3.6	Blood hemolyzed.

Defibrinated sheep blood.

<i>hrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
0	70	27.6	2.5	No hemolysis.
2	61	32.0	2.5	" "
4	42	35.0	2.8	" "
6	40	40.0	2.6	" "
13	19	49.0	2.5	" "
24	13	60.0	3.5	Blood hemolyzed.

TABLE II.

Duration of glycolysis.	Blood.				Remarks.
	Glucose.	Lactic acid.	Inorganic P (serum).	Lipoid P.	
Oxalated dog blood.					
<i>hrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
0	115	30.7	5.2	11.0	No hemolysis.
5	78	36.3		12.0	“ “
10	70	50.0	4.2	11.9	“ “
23	31	61.5	4.8		“ “
30	4	79.8	4.6		“ “

Defibrinated dog blood.

<i>hrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
0	179	48.5	4.5	19.3	No hemolysis.
4	48	63.4	4.5		Slight hemolysis.
6	20	72.0	5.2	19.5	" "
10	16		7.6	19.0	More hemolysis.
24	16	63.0	16.0	19.7	Great hemolysis.

matter of importance in a colorimetric procedure. To 4 volumes of concentrated H_2SO_4 1 volume of the aldehyde-free filtrate was now added cautiously with cooling, to prevent the temperature from rising above 120°C . Running tap water was found more efficient for this purpose than a cooling mixture. When the temperature of the material was reduced to 60°C . the tube was placed in a boiling water bath for 2 minutes. When the solution was completely cooled 1 cc. of a saturated guaiacol solution was added. The pink color developed within 20 minutes. The standard was prepared from pure lactic acid (Merck, 85 per cent) in such a way that 100 cc. of the standard solution contained 20 mg. of the acid. The acid content was checked by analysis.

The inorganic phosphate was determined by the Bell-Doisy method on the whole blood, or by Benedict and Theis' new method when the serum was analyzed. The lipoid P was determined by Bloor's procedure.

Tables I and II give the results which were obtained in some of the longest and most complete series.

The results recorded in Tables I and II show at a glance that, unlike the insulin hypoglycemia, the disappearance of the blood sugar in *in vitro* glycolysis goes parallel with an accumulation of lactic acid. The inorganic phosphorus does not change *provided there is no hemolysis* in the sample. We cannot, therefore, concur in the opinion of Bierry and Moquet that during glycolysis there is a gradual rise in the inorganic P while the lipoid P diminishes in quantity. The few lipoid P determinations which we made on dog's blood fail to support this contention which led Bierry and Moquet to postulate the hypothesis; *viz.*, that preliminary to the transformation of glucose into lactic acid *in vitro* the glucose changes to a hexose-phosphate within the red cells, the lipoids of the latter furnishing the needed phosphorus. According to our experience we believe that such a view is erroneously based upon conditions associated with hemolysis rather than with glycolysis of blood *in vitro*.

CONCLUSION.

The *in vitro* process of glycolysis is different from the hypoglycemia caused by insulin in that the disappearance of the glucose in the former case goes parallel with a formation of lactic acid. The inorganic P remains unchanged during glycolysis, increasing only in blood samples undergoing hemolysis. The increase in P is practically proportional to the degree of hemolysis. There is, therefore, no evidence from the study of *in vitro* glycolysis that

the formation of a hexose-phosphate compound is a preliminary step in the mechanism of the disappearance of the sugar from the blood.

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THE RATE OF ELIMINATION OF INGESTED SUGARS IN PHLORHIZIN DIABETES.

BY H. J. DEUEL, JR., AND W. H. CHAMBERS.*

(From the Department of Physiology, Cornell University Medical College,
New York City.)

(Received for publication, June 24, 1925.)

INTRODUCTION.

The hourly rate of excretion of ingested glucose in phlorhizin diabetes has been reported by Csonka (1), but no experimental work is on record in which a similar study has been made of the rate at which "extra glucose" appears in the urine after the ingestion of fructose or galactose.

The amount of glucose excreted hourly after the administration of monosaccharides or any other potential glucose formers is determined not alone by its rate of absorption from the gut, but also by its rate of conversion into glucose and the rate of elimination by the kidney. If we assume that absorption of fructose or galactose is equally as rapid as that of glucose, the rate of excretion is limited to the latter two factors. In case as rapid an elimination of extra glucose takes place after the administration of fructose or galactose as after glucose, one would assume that the conversion of these monosaccharides to glucose takes place very quickly. On the other hand, a much slower rate of elimination of extra glucose would indicate that the various transformations in the intermediary metabolism of fructose and galactose take an appreciable interval of time. The third consideration, *viz.* the elimination by the kidney, would not affect the rate of excretion of the extra glucose arising from other monosaccharides differently from the way it does that arising from ingested glucose. To answer these questions with regard to the time relations in the intermediary metabolism of fructose and galactose the experiments reported in this paper were undertaken.

* National Research Council Fellow in Medicine (1924-26).

HISTORICAL.

Ringer and Frankel (2) showed that the elimination of extra glucose following administration of glucose took place very quickly, approximately 60 per cent being eliminated 2 hours after its intravenous injection. The extra glucose formed after the injection of the same amount of sodium propionate was much less rapidly eliminated, thus leading these authors to conclude: "*the difference in the time relationship can be attributed only to the time required for the synthesis of glucose from propionic acid.*"

Csonka (1), on the administration of 16 gm. of glucose by mouth to dogs, found an excretion of 46 per cent of extra glucose in 2 hours and the entire amount in 7 hours, while the glucose originating from glycine and alanine was eliminated at practically the same rate. Reilly, Nolan, and Lusk (3) have shown that no fructose was eliminated in the urine as such after feeding 24 gm. to a phlorhizinized dog, but 15.3 gm. of extra glucose originated therefrom, while with rabbits Lusk (4) found some fructosuria following the administration of 20 gm. With galactose, a less satisfactory conversion to glucose was obtained (4), and only 7.9 gm. of extra glucose were found after the ingestion of 24 gm. of this carbohydrate, although no galactosuria was noted. That fructosuria or galactosuria is not to be expected after the oral administration of these sugars to phlorhizinized dogs in the amounts used in the experiments herein reported is indicated from the results of Bodansky (5), who found a galactosuria (as evidenced by a positive mucic acid test) in only two cases out of twenty when 18 to 60 gm. of this sugar were fed to normal dogs. When similar amounts of fructose were given to the same animals he did not obtain a definite fructosuria in a single instance. After subcutaneous administration of this sugar Benedict¹ has found a fructosuria. Brasch (6), on the other hand, did find some galactosuria when much larger amounts of galactose were fed.

Method.

The following experiments were carried out on three fasting phlorhizinized dogs and include two experiments with fructose,

¹ Benedict, S. R., personal communication.

two with glucose, one each with galactose and lactose, and three control experiments in which no food was given.

The dogs were rendered diabetic by the usual technique; *viz.*, the daily subcutaneous injection of 1 gm. of purified phlorhizin in 10 cc. of olive oil. The experimental studies were started when the animals were completely diabetic as judged by the D:N ratio, generally on the 5th day of phlorhizin. The phlorhizin² used in these experiments gave unusually satisfactory results, no intoxication being noted and the animals remaining in good condition for as long as 9 days of phlorhizin and 11 days of fasting. Even better results were obtained with a hog in which no disturbances were evident after the administration of 1.5 gm. of phlorhizin daily for 37 days to the fasting animal.

The urine was collected entirely by catheter at hourly intervals with the few exceptions noted in the protocols. Glucose determinations were carried out by the Benedict method and total nitrogen by the Kjeldahl method. In one experiment on Dog 52 the proportion of fructose excreted as such after its administration was determined. The total reducing power of the urine was first tested and then that of an aliquot portion which had been refluxed for $\frac{1}{2}$ hour with HCl in a concentration of 10 per cent of the acid (3). Control experiments showed that on such treatment fructose is completely decomposed, while no glucose is destroyed, the difference in the titrations before and after refluxing representing the proportion of fructose. Since the glucose determinations were made by Benedict's method it was unnecessary to neutralize the urine after refluxing with 10 per cent of HCl before the titration, as the excess Na_2CO_3 used is adequate.

Calculations of extra glucose were made by multiplying the nitrogen content of the hourly samples by the prevailing D:N

² Phlorhizin is purified as follows: 100 gm. of phlorhizin (Merck) are dissolved in 1000 cc. of 95 per cent alcohol and the solution is filtered, if necessary. To this solution 2800 to 3000 cc. of water are added, with constant stirring, and the mixture is placed in an ice box for several days. The phlorhizin which crystallizes out is filtered off and washed with some distilled water and dried first between filter papers and then in a vacuum desiccator. Heat must be avoided at all stages in this procedure. By this method 88 gm. of purified phlorhizin were obtained from 100 gm. of commercial phlorhizin, the use of which latter sometimes leads to convulsions in the animal.

ratio of the fore period to determine the glucose originating from protein metabolism, and subtracting this value from the total glucose for the hour. The difference represents the extra glucose. To show the rate of excretion on the charts, the extra glucose per hour is expressed in percentage of total extra glucose excreted.

EXPERIMENTAL DATA.

The details of representative individual experiments are given in the protocols and a few typical ones are illustrated in the

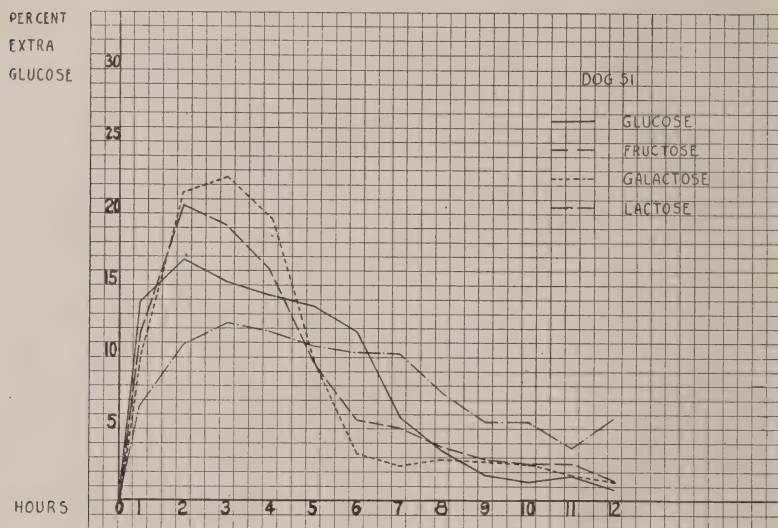


CHART 1. Curves plotted from data of Protocols 1, 2, 3, and 4.

charts. Chart 1 shows the comparative rate of elimination of the four sugars when fed to the same dog.

It is evident that the rate of elimination of extra glucose after feeding 16 gm. of fructose is practically identical with that of glucose itself. The results with galactose suggest a more rapid excretion of extra sugar. This agrees with the statement of Bodansky (5) and Foster (7) that galactose ingestion results in a greater hyperglycemia than that of glucose or fructose. With lactose the extra glucose produced is much less and its excretion

TABLE I.
Rate of Elimination of Extra Glucose after Administration of 16 Gm. of Various Sugars.

Sugar.	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.	7th hr.	8th hr.	9th hr.	10th hr.	11th hr.	12th hr.	Amount recoy- ered.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Glucose.													
Dog 50.....	7.57	16.24	14.66	17.01	10.34	5.57	6.69		21.88*				107
" 51.....	13.88	16.83	15.07	14.13	13.50	11.93	5.78	3.52	1.88	1.32	1.38	0.75	105
Fructose.													
Dog 50.....	11.36	17.63	16.58	14.82	13.84	10.25	6.33	4.24	4.70	0.26			96
" 51.....	11.77	20.58	19.10	16.13	9.57	5.53	4.99	3.51	2.87	2.34	2.34	1.17	93
Galactose.													
Dog 51.....	10.33	21.37	22.51	19.75	9.34	3.04	2.55	2.90	2.76	2.48	1.77	1.20	88
Lactose.													
Dog 51.....	6.29	10.82	12.33	11.83	10.69	10.06	10.06	7.17	5.28	5.16	4.40	5.91	50

* Urine sample for 8th, 9th, and 10th hours.

lasts over a much longer period of time, this extra glucose not having been completely eliminated 12 hours after its ingestion.

The amount of extra glucose eliminated in the urine when glucose, galactose, and fructose were fed represents a practically quantitative recovery, being for glucose 107 and 105 per cent, 96 and 93 per cent with fructose, and 88 per cent with galactose. With lactose, the extra sugar produced in 12 hours was equivalent to only about 50 per cent of the lactose ingested. The experimental results are summarized in Table I. In one control

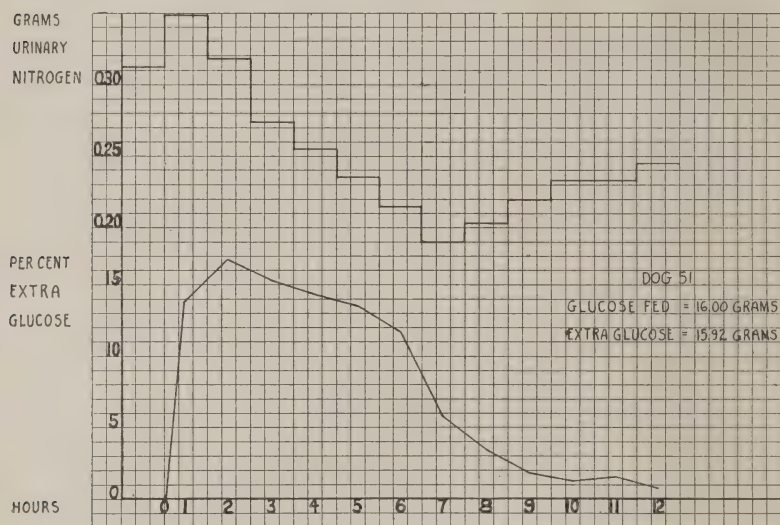


CHART 2. Curves plotted from data of Protocol 1.

experiment on Dog 52, not included in Table I, the amount of fructose eliminated as such was found to be less than 4 per cent of that ingested.

The ingestion of the sugars studied was invariably followed by a sparing action on the nitrogen metabolism despite the fact that the extra glucose formed could not have been oxidized since it was quantitatively excreted in the urine. An example of this is shown in Chart 2, which gives the results of the experiment on feeding glucose to Dog 51. The decrease of protein metabolism becomes evident the 2nd hour after the carbohydrate feeding and contin-

ues until the 7th hour when the minimum value is reached. By this time the bulk of the extra glucose likewise has been excreted and the nitrogen curve rises. In the other experiments, for several hours after the minimum value of nitrogen metabolism is reached (the 6th to the 11th hour) the urinary nitrogen remains at the low figure and then gradually increases. The nitrogen never returns to its former value since the protein catabolism daily becomes smaller, inasmuch as the animals are fasting. Chart 3 shows the 24 hour nitrogen metabolism of Dog 51 during the

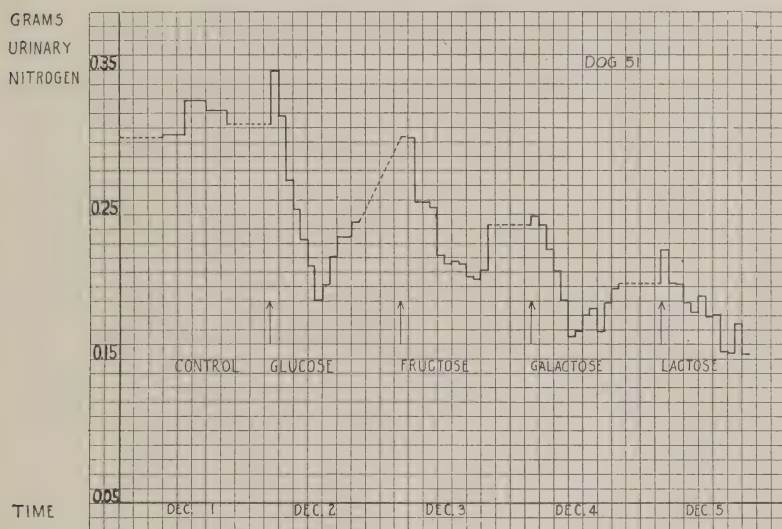


CHART 3. Curves plotted from data of Protocols 1, 2, 3, and 4.

period when the sugars were ingested. The sparing action is evident despite the source of the extra glucose.

Three control experiments in which no food was given show a constant excretion of nitrogen per hour, as illustrated in the protocols. This value was found to be uniform in Dog 56 on January 19 (Protocol 5) with the exception of a high nitrogen excretion during the second hourly period when an increased nitrogen elimination over that of the previous night and of the hours following was concomitant with a considerable diuresis. Diuresis, however, bears no relationship to the decrease in nitrogen excretion

obtained with carbohydrates, since the hourly volume of the urine is considerably increased after carbohydrate ingestion.

It is of interest to note that following the ingestion of sugars the general condition of the animals was markedly improved and their muscular power increased, although the carbohydrates given in these amounts were not burned by the phlorhizinized animals but were quantitatively excreted in the urine.

DISCUSSION.

Our results with glucose are in harmony with those of Ringer and Frankel (2) and Csonka (1) in showing a practically complete elimination in 6 hours, although we have been unable to duplicate Csonka's results in obtaining an elimination of 32 per cent of the extra glucose in the 2nd hour. The highest percentage of extra glucose produced after glucose ingestion was 16.8 per cent in the 2nd hour.

Ringer and Frankel (2), as noted previously, have shown a delayed excretion of extra glucose when sodium propionate was injected intravenously over that produced when glucose was given by vein. They attribute this retardation to the time necessary to bring about a conversion of propionic acid to glucose. Both fructose and galactose are probably broken up into trioses which are then synthesized into glucose. The reactions necessary for a conversion of these sugars into glucose are not of such a complex nature as that involved in the transformation of propionic acid into glucose, since no retardation in excretion over that noted for glucose is found.

This decrease in nitrogen metabolism following glucose ingestion has previously been noted by Ringer (8) and also by Nash and Benedict (9).

SUMMARY.

A study of the hourly elimination of extra glucose after the ingestion of 16 gm. of glucose, fructose, and galactose in fasting phlorhizinized dogs showed rates practically identical. Lactose, on the other hand, was excreted much more slowly, and less than 50 per cent of extra glucose arising therefrom was recovered at the end of 12 hours.

The glucose originating from fructose represents a 100 per cent conversion from the carbohydrate ingested and was almost completely eliminated in 5 hours.

The conversion of galactose to glucose has been shown to be nearly quantitative, 88 per cent of the theoretical amount being excreted as extra glucose.

A sparing action on nitrogen metabolism has been noted in each case in which there was increased glucose excretion irrespective of its source, although the glucose was not utilized and was quantitatively excreted.

The authors wish to thank Professor Graham Lusk for the suggestion of the problem and for his helpful criticism.

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PROTOCOL 1.
Elimination of Glucose in Hourly Periods Following the Ingestion of 16 Gm. of Glucose.
 Dog 51. Weight, 10.3 kilos.

Date.	End of period.	Glucose.	Nitro- gen per hr.	D:N ratio.	Extra glucose.		Remarks.
					gm.	per cent	
1924 Nov. 30- Dec. 1	9.00 a.m. (23 hrs.)	24.29	0.302	3.50			Dog fasted Nov. 26 and 1 gm. phlorhizin given subcutaneously daily beginning Nov. 27.
	12 m.	3.55	0.304	3.89			
	3 p.m.	3.82	0.329	3.87			
	6 "	3.80	0.320	3.95			
Dec. 2	9.00 a.m. (15 hrs.)	17.26	0.312	3.67			16 gm. glucose at 9 a.m.
	10 a.m.	3.49	0.349	10.00	2.21	13.88	
	11 "	3.85	0.318	12.11	2.68	16.83	
	12 m.	3.41	0.274	16.17	2.40	15.07	
	1 p.m.	3.18	0.254	12.52	2.25	14.13*	
	2 "	3.01	0.233	12.92	2.15	13.50	
	3 "	2.69	0.214	12.57	1.90	11.93	
	4 "	1.62	0.190	8.53	0.92	5.78	
	5 "	1.30	0.202	6.46	0.56	3.52	
	6 "	1.11	0.220	5.06	0.30	1.88	
	7 "	1.07	0.234	4.57	0.21	1.32	
	8 "	1.08	0.234	4.62	0.22	1.38	
	9 "	1.01	0.245	4.12	0.12	0.75	
" 3	Unknown.	1.70	0.457	3.72	15.92		Morning bladder sample.

* Dog vomited 0.888 gm. glucose, therefore 15.11 gm. were retained.

PROTOCOL 2.
Elimination of Glucose in Hourly Periods Following the Ingestion of 16 Gm. of Fructose.
 Dog 51.* Weight, 10.3 kilos.

Date.	End of period.	Glucose.		Nitrogen per hr.	D:N ratio.	Extra glucose.		Remarks.
		gm.	gm.			gm.	per cent	
1924 Dec. 3	Unknown.	1.70	0.457	3.72				
	11 a.m. (1 hr.)	2.64	0.304	8.68		1.51	11.77	Night urine lost. That collected represents bladder urine in period previous to start of experiment. 16 gm. fructose given at 10.00 a.m. Dog vomited. Fructose content = 1.08 gm. Therefore fructose fed = 16.00-1.08 = 14.92 gm.
	12 m.	3.78	0.303	12.47		2.65	20.58	
	1 p.m.	3.41	0.259	13.17		2.45	19.10	
	2 "	3.03	0.259	11.70		2.07	16.13	
	3 "	2.19	0.255	8.59		1.24	9.57	
	4 "	1.54	0.221	6.97		0.71	5.53	
	5 "	1.43	0.216	6.62		0.63	4.99	
	6 "	1.26	0.218	5.78		0.45	3.51	
	7 "	1.17	0.214	5.47		0.37	2.87	
	8 "	1.07	0.207	5.17		0.30	2.34	
	9 "	1.07	0.205	5.22		0.30	2.34	
	10 "	0.94	0.212	4.43		0.15	1.17	
" 4	9.30 a.m. (11½ hrs.)	9.15	0.242	3.36		13.83		

* For previous data on this dog see Protocol 1.

PROTOCOL 3.
Elimination of Glucose in Hourly Periods Following the Ingestion of 16 Gm. of Galactose.
 Dog 51.* Weight, 10.3 kilos.

Date.	End of period.	Glucose.	Nitro- gen per hr.	D:N ratio.	Extra glucose.		Remarks.
		gm.	gm.		gm.	per cent	
1924 Dec. 4	9.30 a.m. (11 $\frac{1}{4}$ hrs.)	9.15	0.242	3.36			16 gm. galactose given at 9.30 a.m.
	10.30 a.m.	2.30	0.249	8.87	1.46	10.33	
	11.30 "	3.83	0.242	15.83	3.02	21.37	
	12.30 p.m.	3.94	0.226	17.43	3.18	22.51	
	1.30 "	3.50	0.210	16.67	2.79	19.75	
	2.30 "	1.96	0.190	10.32	1.32	9.34	
	3.30 "	0.98	0.164	5.98	0.43	3.04	
	4.30 "	0.93	0.169	5.50	0.36	2.55	
	5.30 "	1.02	0.181	5.63	0.41	2.90	
	6.30 "	1.01	0.184	5.49	0.39	2.76	
	7.30 "	0.92	0.169	5.44	0.35	2.48	
	8.30 "	0.88	0.188	4.68	0.25	1.77	
	9.30 "	0.84	0.198	4.24	0.17	1.20	
	" 5 9.30 a.m. (12 hrs.)	8.54	0.203	3.33			
						14.13	

* For previous data on this dog see Protocols 1 and 2.

PROTOCOL 4.

Elimination of Glucose in Hourly Periods Following the Ingestion of 16 Gm. of Lactose.

Dog 51.* Weight, 10.3 kilos.

Date.	End of period.	Glucose.	Nitro- gen per hr.	D: N ratio.	Extra glucose.		Remarks.
		gm.	gm.		gm.	per cent	
1924 Dec. 5	9.30 a.m. (12 hrs.)	8.54	0.203	3.33			16 gm. lactose at 9.30 a.m.
	10.30 a.m.	1.25	0.225	5.55	0.50	6.29	
	11.30 "	1.53	0.203	7.54	0.86	10.82	
	12.30 p.m.	1.65	0.202	8.17	0.98	12.33	
	1.30 "	1.57	0.189	8.31	0.94	11.83	
	2.30 "	1.46	0.182	8.02	0.85	10.69	
	3.30 "	1.45	0.194	7.47	0.80	10.06	
	4.30 "	1.39	0.179	7.77	0.80	10.06	
	5.30 "	1.17	0.181	6.46	0.57	7.17	
	6.30 "	0.93	0.155	6.00	0.42	5.28	
	7.30 "	0.92	0.154	5.97	0.41	5.16	
	8.30 "	0.94	0.176	5.34	0.35	4.40	
	9.30 "	1.04	0.153	6.80	0.47	5.91	
					7.95		

* For previous data on this dog see Protocols 1, 2, and 3.

PROTOCOL 5.

*Elimination of Glucose and Nitrogen in Hourly Periods in a Fasting
Phlorhizinized Dog.*

Dog 56. Weight, 9.8 kilos.

Date.	Period.	Glucose.	Nitrogen.		D:N ratio.
			Per period.	Per hr.	
<i>1925</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Jan. 18-19	7.45 p.m.- 9.17 a.m.	17.67	4.754	0.351	3.72
	9.17 a.m.-10.18 "	1.46	0.370	0.364	3.94
	10.18 " -11.17 "	1.58	0.408	0.415	3.87
	11.17 " -12.19 p.m.	1.50	0.383	0.372	3.92
	12.19 p.m.- 1.21 "	1.49	0.387	0.375	3.85
	1.21 " - 2.22 "	1.50	0.377	0.371	3.98
	2.22 " - 6.26 "	5.27	1.418	0.348	3.72
" 19-20	6.26 " - 9.25 a.m.	18.66	5.395	0.360	3.46

ANIMAL CALORIMETRY.

THIRTIETH PAPER.

THE METABOLISM OF GLYCEROL IN PHLORHIZIN DIABETES.

BY W. H. CHAMBERS* AND H. J. DEUEL, JR.

*(From the Department of Physiology, Cornell University Medical College,
New York City.)*

(Received for publication, June 24, 1925.)

Glycerol is ordinarily listed with the intermediary products of carbohydrate metabolism, which can be completely converted into glucose in the diabetic organism, although no published data showing its total recovery as urinary sugar are to be found. Hirschfeld (1), Satta (2), and Lang (3) have reported a decrease in acetonuria after giving glycerol to diabetic and to normal fasting individuals. Voegtlin, Dunn, and Thompson (4) have shown that glycerol will replace glucose in relieving the hypoglycemia and toxic effects of insulin injections. Cremer (5) found that about 40 per cent of the glycerol ingested by a phlorhizinized dog was excreted in the urine as "extra glucose," and Lüthje (6) obtained similar results with depancreatized dogs. Recently McCann, Hannon, Perlzweig, and Tompkins (7) and Thomas (8) have used this conversion figure of 40 per cent as a basis for calculating the antiketogenic value of glycerol in the diets of diabetic patients. To determine more exactly the quantitative conversion of glycerol into glucose, experiments were made on phlorhizinized dogs in which the urine was collected at hourly intervals.

EXPERIMENTAL DATA.

The technique of phlorhizinizing, collecting the urine, and calculating the results is published in a previous paper (9). Glycerol, U.S.P., was given by stomach tube in aqueous solution or injected subcutaneously. The specific gravity of the glycerol, determined

* National Research Council Fellow in Medicine (1924-26).

by pycnometer, was 1.2497 which, according to the table in "Watts' Dictionary of chemistry" (10), equals by interpolation 94.76 per cent. It should be noted in calculating the recovery of glycerol as glucose that 2 molecules of glycerol yield 1 molecule of glucose and 4 atoms of hydrogen



or 100 gm. of glycerol yield 97.8 gm. of glucose and 2.2 gm. of hydrogen.

The complete data of five experiments made on the three female dogs are given in the protocols. In the first experiment (Dog 56, January 20, Protocol 1) 15.16 gm. of glycerol were ingested and only 55.9 per cent were recovered in the urine as extra glucose.

TABLE I.
Summary.

Dog No.	Glycerol ingested.	Glucose equivalent.	Glucose recovered.	
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
56	15.16	14.85	8.30	55.9
56	8.53*	8.33	5.83	70.0
56	8.53	8.33	4.46	53.8
57	8.53	8.33	8.20	98.4
51	8.53	8.33	8.07	96.9

* Subcutaneous injection.

The following day into the same dog a smaller amount, 8.53 gm., was injected subcutaneously to eliminate the factor of absorption. The yield of extra glucose was 70 per cent. When the same amount, 8.53 gm., was ingested the next day the return was 53.8 per cent.

The other two experiments with Dogs 57 and 51 are given in Protocols 2 and 3. From 8.53 gm. of ingested glycerol 8.20 and 8.07 gm. of extra glucose were obtained in the urine, a yield of 98.4 and 96.9 per cent, respectively. These results relating to the amount of glycerol converted into glucose are summarized in Table I.

In two of the dogs (Dogs 57 and 51) there was an almost complete recovery of the glycerol in the form of glucose, while the third dog (Dog 56) yielded about 60 per cent. The metabolism

of the last dog was therefore determined in the respiration calorimeter to see if there was an oxidation of the unrecovered portion of glycerol.

On the day following the experiments tabulated in Protocol 1 the basal metabolism of Dog 56 was determined for 2 consecutive hours, 8.53 gm. of glycerol were then ingested and the metabolism was again observed for the succeeding 2nd, 3rd, and 4th hours. The data are given in Table II.

The respiratory quotients average 0.703 for the basal metabolism and 0.678 for the 3 hours after the glycerol ingestion. There is a slight decrease in the heat production of questionable sig-

TABLE II.

Metabolism of Dog 56 before and after Ingesting Glycerol in Phlorhizin Glycosuria.

Hourly period ending.	Experiment.	CO ₂	O ₂	R.Q.	N	Non-protein R.Q.	Total calories.
<i>p.m.</i>		<i>gm.</i>	<i>gm.</i>		<i>gm.</i>		
3.41	Basal metabolism.	5.84	6.25	0.680	0.304	0.691	19.47
4.41		6.10	6.10	0.727	0.304	0.754	20.34
Averages.		5.97	6.18	0.703	0.304	0.723	19.91
7.14	After ingesting 8.53 gm. glycerol at 4.59 p.m.	5.28	5.79	0.663	0.293	0.672	18.42
8.14		5.22	5.41	0.702	0.312	0.724	18.20
9.14		5.69	6.17	0.671	0.312	0.680	19.77
Averages.		5.40	5.79	0.678	0.306	0.692	18.80

nificance, as the averages are 19.91 calories before and 18.80 calories after taking the glycerol.

DISCUSSION.

Our experiments on three dogs indicate that a complete conversion of glycerol into glucose is found in some dogs and that the lower figures of earlier workers might be explained on the basis of the difference in individual animals. A 70 per cent recovery on subcutaneous injection and one of 55 per cent after ingestion in Dog 56 suggest that an incomplete absorption may account for a part of the unrecovered portion of the glycerol. The calorimeter

experiment excludes the oxidation of the glycerol as an explanation for the unrecovered balance.

The decrease in the respiratory quotient from a basal of 0.703 to 0.678 on the ingestion of glycerol (Table II) is a significant one. As shown in the equation above, the conversion of 2 molecules of glycerol to 1 of glucose yields in addition 4 atoms of hydrogen. The recovery of extra glucose was not determined in the calorimeter experiment, but on the previous day (January 22, Protocol 1) 3.53 gm. of extra glucose were excreted during the 2nd, 3rd, and 4th hours after giving *per os* the same amount of glycerol, 8.53 gm. If oxidized, the hydrogen from the amount of glycerol converted into glucose requires 0.628 gm. or 0.440 liter of additional oxygen. If this be subtracted from the oxygen used in the oxidation of the non-protein fraction of the total metabolism, one may calculate the respiratory quotient of the rest of the material oxidized. The formula is as follows:

$$\text{R.Q.} = \frac{6.37 \text{ liters of CO}_2}{9.23 - 0.44 \text{ liters of O}_2} = 0.725$$

This agrees almost exactly with the non-protein quotient of the preceding period (0.723) in which no glycerol was given. Since the respiratory quotient of glycerol is 0.857, it follows that glycerol itself could not have been oxidized.

One must consider that when 3.53 gm. of glucose (13.24 calories, Rubner) are produced from 3.61 gm. of glycerol (15.57 calories, Stohmann), 2.33 calories are liberated in the reaction. One also may estimate that, if the hydrogen liberated in this reaction united directly with oxygen, 2.72 calories would have been produced. Therefore approximately one-third of 2.33 calories, or 0.80 calorie, has been added to the computed hourly heat production in this part of the experiment. The results of these metabolism experiments, therefore, indicate that in the phlorhizinized dog there may be a complete conversion of glycerol to glucose and no detectable oxidation of the glycerol or the glucose which is formed from it, except as to the hydrogen liberated in the reaction.

McCann and Hannon (11) have reported a decrease in the

respiratory quotient after the ingestion of glycerol by diabetic patients. However, they have found a similar effect in the same patients when glucose was taken.

The hourly rate of excretion of ingested glycerol is practically the same as that of the monosaccharides. Thus, the same dog (Dog 51) which was used in both kinds of experiments gave almost identical figures for the percentage of extra glucose eliminated during the first 3 hours after the administration of both glycerol (Protocol 3) and galactose.¹ From the figures of hourly nitrogen excretion given in the protocols it is seen that glycerol produces a sparing of the protein metabolism in the same manner as do the monosaccharides.

No gastrointestinal disturbances were apparent following the administration of the glycerol in the small amounts used in these experiments.

SUMMARY.

A practically complete conversion of glycerol to glucose was exhibited by two phlorhizinized dogs. After the ingestion of 8.53 gm. of glycerol, 96.9 and 98.4 per cent, respectively, of extra glucose were recovered in the urine.

A third dog yielded 55 per cent conversion after ingestion and 70 per cent after subcutaneous injection of glycerol. A decrease in the respiratory quotient from 0.703 to 0.678 after giving glycerol indicates that there was no general oxidation of the ingested glycerol, which has a respiratory quotient of 0.86, nor of the glucose into which it was converted. The reduction in the respiratory quotient from 0.703 to 0.678 may be exactly accounted as due to the oxidation of the 2 atoms of hydrogen liberated in the reaction $\text{glycerol} \rightarrow 1/2 \text{ glucose}$.

In the rate of excretion and the sparing of protein metabolism the reaction of glycerol was similar to that of the monosaccharides.

To Professor Graham Lusk the authors are indebted for his kindly suggestions and criticism.

¹ Deuel and Chambers (9), Protocol 3, p. 18.

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PROTOCOL 1.

Glucose and Nitrogen Elimination during Glycerol Experiments.

Dog 56. Weight, 9.8 kilos.

Date.	Time.	Glucose.	Nitrogen.		D:N ratio.	Extra glucose.		Remarks.
			Per period.	Per hr.				
1925		gm.	gm.	gm.		gm.	per cent	
Jan. 18	7.45 p.m.							
" 19	9.17 a.m.	17.67	4.754	0.351	3.72			
	10.17 "	1.44	0.364	0.364	3.94			
	11.17 "	1.60	0.415	0.415	3.87			
	12.17 p.m.	1.45	0.372	0.372	3.92			
	1.17 "	1.44	0.375	0.375	3.85			
	2.17 "	1.48	0.371	0.371	3.98			
	6.26 "	5.40	1.448	0.348	3.73			
" 20	9.25 a.m.	18.66	5.495	0.360	3.30 3.55*			
	10.25 "	1.09	0.340	0.340	3.21			
	11.25 "	1.15	0.346	0.346	3.31			
	12.33 p.m.	2.17	0.438	0.388	5.04	0.64	7.71	15.16 gm. glycerol ingested at 11.33 a.m.
	1.33 "	2.60	0.301	0.301	8.64	1.55	18.66	
	2.33 "	2.85	0.260	0.260	10.96	1.94	23.38	
	3.33 "	2.39	0.215	0.215	11.11	1.44	17.35	
	4.33 "	2.07	0.254	0.254	8.15	1.18	14.22	
	5.33 "	1.54	0.257	0.257	5.99	0.64	7.71	
	6.33 "	1.19	0.238	0.238	5.00	0.36	4.34	
	7.33 "	1.01	0.240	0.240	4.21	0.17	2.05	
	8.33 "	1.00	0.236	0.236	4.23	0.17	2.05	
	9.33 "	1.05	0.276	0.276	3.80	0.04	0.48	
	10.33 "	1.17	0.287	0.287	4.07	0.17	2.05	
						8.30		
" 21	9.33 a.m.	10.93	2.894	0.263	3.78 3.68*			
	11.49 "	2.53	0.609	0.269	4.15			8.53 gm. glycerol injected subcutaneously at 11.56 a.m.
	12.56 p.m.	2.18	0.379	0.340	5.75	0.72	12.36	
	1.56 "	2.53	0.295	0.295	8.58	1.40	24.01	
	2.56 "	2.85	0.293	0.293	9.73	1.72	29.50	
	3.56 "	2.63	0.324	0.324	8.12	1.39	23.84	
	4.56 "	1.69	0.351	0.351	4.81	0.34	5.83	
	5.56 "	1.37	0.308	0.308	4.45	0.19	3.26	
	6.56 "	1.20	0.294	0.294	4.08	0.07	1.20	
						5.83		
	7.56 "	1.13	0.306	0.306	3.69			
	11.01 "	3.14	0.845	0.274	3.72			

PROTOCOL 1—*Concluded.*

Date.	Time.	Glucose.	Nitrogen.		D:N ratio.	Extra glucose.		Remarks.
			Per period.	Per hr.				
1925		gm.	gm.	gm.		gm.	per cent	
Jan. 22	9.51 a.m.	12.99	3.322	0.307	{ 3.92 3.85*			8.53 gm. glycerol ingested at 9.55 a.m.
	11.55 "	4.10	0.605	0.294		1.77	39.68	
	1.55 p.m.	4.60	0.623	0.312	7.38	2.20	49.33	
	3.55 "	2.49	0.517	0.259	4.82	0.49	10.99	
						4.46		
	5.55 "	2.04	0.572	0.286	3.57			
" 23	11.53 a.m.	19.48	5.192	0.288	3.75			

* The last portion of the night sample was collected by catheter and the D:N ratio determined separately.

PROTOCOL 2.

Hourly Elimination of Glucose after the Ingestion of Glycerol.

Dog 57. Weight, 7.8 kilos.

Date.	Time.	Glucose.	Nitrogen.		D:N ratio.	Extra glucose.		Remarks.
			Per period.	Per hr.				
1925		gm.	gm.	gm.		gm.	per cent	
Feb. 2	2.29 p.m.							
" 3	9.13 a.m.	19.62	4.992	0.266	{ 3.93 3.97*			8.53 gm. glycerol ingested at 9.17 a.m.
	10.17 "	2.37	0.312	0.288		1.13	13.78	
	11.17 "	3.02	0.238	0.238	12.69	2.08	25.37	
	12.17 p.m.	2.51	0.213	0.213	11.78	1.66	20.24	
	1.17 "	1.54	0.174	0.174	8.85	0.87	10.61	
	2.17 "	1.37	0.215	0.215	6.37	0.52	6.34	
	3.17 "	1.25	0.208	0.208	6.01	0.42	5.12	
	4.17 "	1.12	0.200	0.200	5.60	0.33	4.02	
	5.17 "	1.16	0.220	0.220	5.27	0.29	3.54	
	6.17 "	1.04	0.208	0.208	5.00	0.21	2.56	
	7.17 "	0.92	0.214	0.214	4.30	0.07	0.85	
	8.17 "	0.97	0.222	0.222	4.37	0.09	1.10	
" 4	9.01 a.m.	11.83	2.864	0.225	{ 4.13 3.62*	0.53	6.47	
						8.20		

* The last portion of the night sample was collected by catheter and the D:N ratio determined separately.

PROTOCOL 3.

Hourly Elimination of Glucose after the Ingestion of Glycerol.

Dog 51. Weight, 8.42 kilos.

Date.	Time.	Glucose.	Nitrogen.		D:N ratio.	Extra glucose.		Remarks.
			Per period.	Per hr.				
1925		gm.	gm.	gm.		gm.	per cent	
Jan. 27	11.19 a.m.							
	5.19 p.m.	7.12	1.545	0.258	4.61			
" 28	9.08 a.m.	14.77	3.718	0.235	(4.20			8.53 gm. glycerol ingested at 9.33 a.m.
					3.88*			
	10.33 "	2.23	0.364	0.258	8.64	0.82	10.16	
	11.33 "	2.80	0.276	0.276	10.14	1.73	21.44	
	12.33 p.m.	2.70	0.228	0.228	11.84	1.82	22.55	
	1.33 "	1.74	0.194	0.194	8.97	0.99	12.27	
	2.33 "	1.46	0.222	0.222	6.58	0.60	7.43	
	3.33 "	1.18	0.203	0.203	5.81	0.39	4.83	
	4.33 "	1.15	0.228	0.228	5.04	0.27	3.35	
	5.33 "	1.24	0.237	0.237	5.23	0.32	3.97	
" 29	9.58 a.m.	15.37	3.684	0.224	(4.21	1.13	14.00	
					3.67*	8.07		

* The last portion of the night sample was collected by catheter and the D:N ratio determined separately.

LACTONE FORMATION FROM MONO- AND DICARBOXYLIC SUGAR ACIDS.

BY P. A. LEVENE AND H. S. SIMMS.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

The most recent phase of the work on simple carbohydrates is concerned with isomerisms occasioned by differences in size of the oxidic ring. The first observations which brought to light the existence of such isomers were made by Nef¹ and by E. Fischer.² Nef discovered two isomeric galactonic lactones and Fischer a third isomer of 1-methyl glucose. Isomers of this type have been discovered for the derivatives of many monosaccharides through the efforts of Irvine³ and of other workers of his school, through those of Hudson⁴ and others. Very recently isomers of this type have been described for lactones of methylated galactonic acid (Pryde and coworkers⁵ and Haworth and coworkers⁶) and of methylated mannonic acid (Levene and Meyer⁷).

Fischer and Irvine have noted that the new isomeric 1-methyl hexoses were less "stable" than the common forms and that carbon atom (1) in the new forms is more reactive than the corresponding atom in the common forms. From the observations of Haworth, Ruell, and Westgarth it follows that tetramethyl galactonic-1, 4-lactone is more "stable" than its 1,5-isomer. One of the two tetramethyl mannonic lactones prepared by Levene and Meyer

¹ Nef, J. U., *Ann. Chem.*, 1914, cdiii, 331.

² Fischer, E., *Ber. chem. Ges.*, 1914, xlvii, 1980.

³ Irvine, J. C., Fyfe, A. W., and Hogg, T. P., *J. Chem. Soc.*, 1915, cvii, 524.

⁴ Hudson, C. S., *J. Am. Chem. Soc.*, 1915, xxxvii, 1591. Hudson, C. S., and Johnson, J. M., *J. Am. Chem. Soc.*, 1916, xxxviii, 1223.

⁵ Pryde, J., Hirst, E. L., and Humphreys, R. W., *J. Chem. Soc.*, 1925, cxxvii, 348.

⁶ Haworth, W. N., Ruell, D. A., and Westgarth, G. C., *J. Chem. Soc.*, 1925, cxxv, 2468.

⁷ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1924, lx, 167.

was more "stable"⁸ than the other. In the case of mannonic lactones the decision regarding the structure of the isomers was arbitrary because there was no rational basis for differentiation of the isomeric sugar acids having lactones which rotate in the same direction.

It therefore seemed advisable to undertake a quantitative study of the rates (and extent) of formation and splitting of isomeric lactones of known structure in order to find a rational basis for assigning structures to isomeric lactones which rotate in the same direction.

Another reason for the present investigation is that the structural models of isomeric normal sugar acids indicate that all these acids should form lactones of the same type at nearly equal rates. This should be true of monocarboxylic and also of dicarboxylic sugar acids. This appears to be contrary to our experience that certain of these acids are more readily isolated in the lactone form than are others.

DISCUSSION.

A. Monocarboxylic Acids.—Considering first the monocarboxylic acids (of the *d* series) we see that each is capable of forming a lactone with a 5-membered ring and also one with a 6-membered ring. (The 7-membered ring is improbable.) We should expect both lactones to form simultaneously, but at different rates, depending upon the energy changes involved in the respective linkages.⁹ For convenience, we shall refer to these lactones as "5-ring lactones" and "6-ring lactones," respectively.

⁸ In acidic solutions at the same pH, the lactone which hydrolyzes with greater initial velocity continues to a greater percentage of hydrolysis at equilibrium, and is referred to as being "less stable." The word "stable" should, however, be used with discretion since a reaction with a greater initial velocity does not always continue to an equilibrium at which a greater amount is changed. This is the case with the reverse reaction in which the corresponding acids are allowed to form lactones. Hence, we arbitrarily define our use of the word "stable" as referring to the initial velocity of reaction, rather than to the state of equilibrium.

⁹ The linkages involving the hydroxyl groups on carbon atoms (4) and (5), respectively, may be expected to involve different energy changes owing to the greater proximity of the carboxyl group to the former. The different mechanical probabilities of the formation of a 5-membered or a 6-membered ring may also affect the results.

We find that gulonic acid (*A*), galactonic acid (*B*), glucoheptonic acid (*C*), and mannonic acid (*D*), in fourth molar solutions of the pure acids at 25° C., all form a small amount (30 per cent or less) of 6-ring lactone, reaching equilibrium within a few hours. At the same time, there is a much slower reaction, requiring several hundred hours to reach equilibrium, in which a larger

TABLE I.

Approximate Initial Reaction Rates in Formation (k_f) and Splitting (k_s) of Lactones.

	Monocarboxylic acids (data from gulonic acid).		Dicarboxylic acids (estimated). (Resultant effect.)
	6-ring lactone.	5-ring lactone.	
Time to reach one-half equilibrium, hrs..	1.5	38	
Lactone at equilibrium, per cent.....	25	80	(75)
$k_f \times 290$	32	4	(1-2)
$k_s \times 290$	100	1	(1/3-1/2)

TABLE II.

Monocarboxylic Acids Capable of Forming Only One Lactone.

Acid.	Letter.	Type of ring.	Lactone at equilibrium.	Time to reach one-half equilibrium.	Reaction rates.	
					$k_f \times 290$	$k_s \times 290$
			per cent	hrs.		
4-Methyl glucoheptonic.	(E)	6-membered.	21	2.5	17	64
2, 3, 4, 6-tetramethyl mannonic.....	(F)	6- "	30	6	10	23
2, 3, 5, 6-tetramethyl mannonic.....	(G)	5- "	75	117	1.3	0.4

amount (about 80 per cent) of 5-ring lactone is formed. The initial velocity of the former reaction is eight times that of the latter (equation (5)).

We offer no explanation of the anomaly that the lactone which forms more readily is produced in smaller amount. The relations are shown in Table I, in which the rates of reaction were calculated from equation (5). The values in the second and third columns

were obtained from gulonic acid (*A*) (Table VI and Fig. 2) and are approximately correct for all the monocarboxylic acids.

We corroborated this by measuring the lactone formation of certain substituted acids which could form only a single lactone, as shown in Table II.

The two tetramethyl acids are weaker acids and their reaction rates are relatively the same, although somewhat slower than the non-methylated acids.

The rates of lactone formation of the monocarboxylic sugar acids were measured by means of their optical rotation. If $[M_A]$ is the molecular rotation of the pure acid and $[M_L]$ is the molecular rotation of the pure lactone, then the percentage of lactone formation when the observed molecular rotation is $[M]$ is

$$\pm \text{ per cent} = 100 \frac{[M] - [M_A]}{[M_L] - [M_A]} \quad (1)$$

We shall designate the lactone formation as *positive* when the lactone is *more dextro* than the acid, and as *negative* when it is *more levo* than the acid.

In Fig. 1 the three substances (*E*), (*F*), and (*G*) which form but a single lactone have their lactone formation accurately plotted within experimental error.

The curves for those substances (*A*, *B*, *C*, and *D*) which form two lactones are plotted on the basis of the value of $[M_L]$ for the 5-ring lactone. If the numerical value of $([M_L] - [M_A])$ is the same for the 6-ring lactone of an acid as it is for the 5-ring lactone (regardless of sign), then the curves represent the *true* percentages of "6-ring lactone minus 5-ring lactone." If, however, the change in molecular rotation is not the same in the formation of the two lactones, then the curves only qualitatively represent the conditions. (These changes are probably of the same order of magnitude.)

From the structural formulas (and models) it is obvious that all the monocarboxylic sugar acids of the *d* series have the same (*dextro*) configuration of carbon atom (5). The formation of a lactone with the hydroxyl on this carbon atom should *increase* the *dextro* rotation of the molecule. This is true. Fig. 1 shows that all the 6-ring lactones are *dextro* with respect to the acid (*i.e.*, all the curves are *positive* at the start).

Carbon atom (4), on the other hand, is *levo* in half of the acids (talonic, galactonic, idonic, and gulonic) while the other half (mannonic, gluconic, altronic, and allonic) have a *dextro* carbon atom (4). Hence, the 5-ring lactones of the first half should be more *levo* and the others more *dextro* than the free acid. This is also true. The mannonic acids (*D*) and (*G*) are *dextro* in forming 5-ring lactones while gulonic (*A*) and galactonic (*B*) are *levo*.

Likewise, for the heptonic acids, the 4-methyl glucoheptonic acid (*E*) gives a *dextro* 6-ring and glucoheptonic acid (*C*) gives a *dextro* 6-ring and a *levo* 5-ring, in entire agreement with the structural formulas.

In Fig. 1 the small circles represent points determined in 0.250 molar solution of free acid. However, the large circles represent points obtained in the presence of an equivalent of hydrochloric acid. From the dissociation constant of gulonic acid ($10^{-3.68}$, corrected for activity), we calculated that the activity of hydrogen ion in the second solution was 33 times that in the first. Multiplying the time (*t*) in the second experiment by 33, places the two sets of points on essentially the same curves. Since the same factor (33) holds for galactonic and glucoheptonic acids (Curves *B* and *C*, respectively), we may assume that they have essentially the same dissociation constants as gulonic acid (as would be expected).

An experiment was performed on gulonic acid in which not only the rotation, but also the volumetric titration with NaOH, were measured. The results are represented in Fig. 2. The rotation data give Curve I, Fig. 2 (which is essentially the same as Fig. 1, *A*). This is the resultant curve of the formation of a positive 6-ring lactone and a negative 5-ring lactone. The titration data give values corresponding approximately to the quantity of 5-ring lactone (the 6-ring lactone is largely hydrolyzed in the titration). This is shown in Curve II. The difference between these gives Curve III for the amount of 6-ring lactone present at various stages of the reaction. The drop in Curve III is expected since 6-ring lactone is in equilibrium with a constantly decreasing amount of free acid.

In order to find the curve which would be observed (optically) if the 5-ring lactone were positive instead of negative, we may add (numerically) the values in Curve II to those in Curve III. This

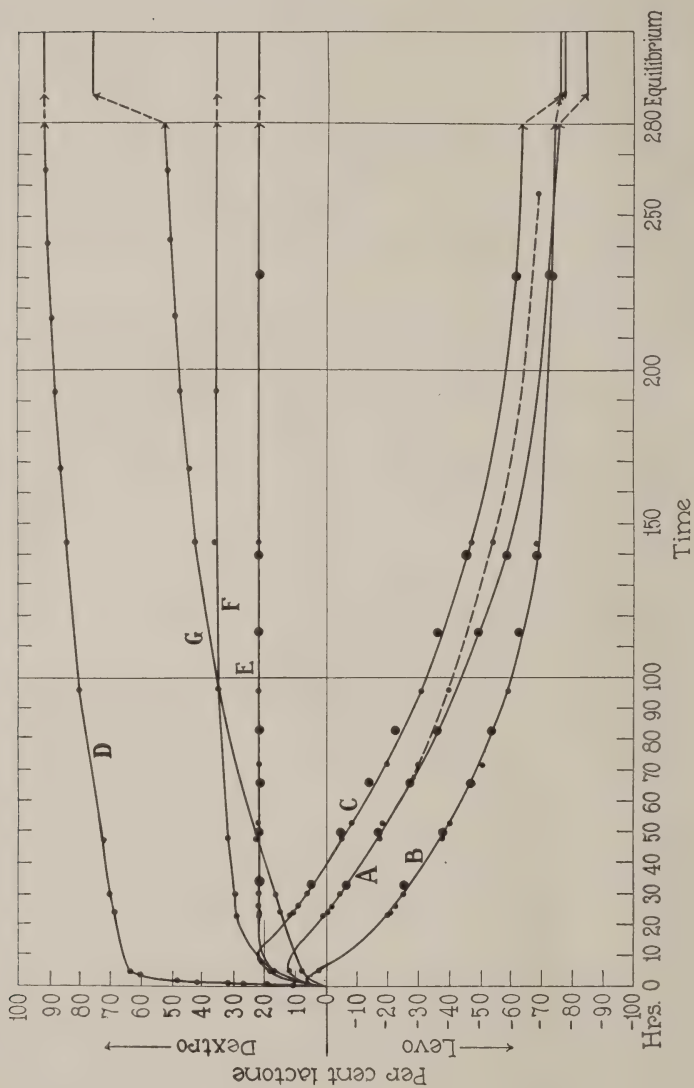


Fig. 1.

FIG. 1. Lactone formation of monocarboxylic acids at 25°C.

Letter.	Acid.	Types of lactones.
(A)	Gulonic.	} +6-Ring and -5-ring.
(B)	Galactonic.	
(C)	Glucoheptonic.	
(D)	Mannonic.	+6-Ring and +5-ring.
(E)	4-Methyl glucoheptonic.	+6-Ring.
(F)	2,3,4,6-Tetramethyl mannonic.	"
(G)	2,3,5,6-Tetramethyl mannonic.	+5-Ring.

The circles are omitted below 5 hours (except in Curve D).

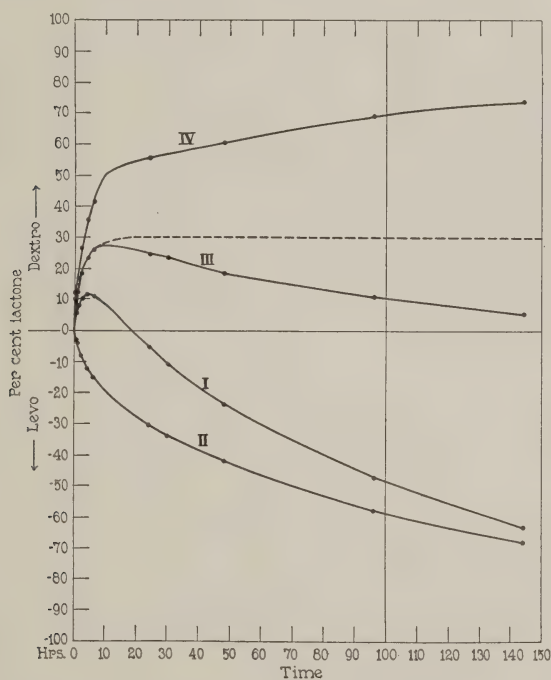


FIG. 2. Gulonic acid at 25°C.

- (I) +6-Ring and -5-ring lactones (observed by rotation).
 (II) -5-Ring lactone (observed by titration).
 (III) +6-Ring lactone
 (IV) +6-Ring and +5-ring lactones } Calculated from data of Curves I and II.
 (Curve IV is hypothetical for gulonic acid. Compare with Fig. 1, D).

gives us Curve IV which is essentially like Fig. 1, *D* for mannonic acid—as is to be expected.

The above reactions should be monomolecular at constant pH. Since they approach an equilibrium in which a fraction *A* is in lactone form, we may express the reaction by the formula¹⁰

$$k_f t = A \ln \frac{A}{A - X} = \frac{K}{K + 1} \ln \frac{A}{A - X} \quad (5)$$

where a fraction *X* is changed in time *t*, and *k_f* is the constant for lactone formation while *K* is the equilibrium constant. Owing to the complexity of dealing with two reactions which approach different equilibria, we have not attempted to apply equation (5) to all our data but merely to gulonic acid (*A*) and to (*E*), (*F*), and (*G*). (See approximate values in Tables I and II.)

B. Dicarboxylic Acids.—The dicarboxylic acids derived from hexoses can form either of two 6-ring lactones or either of two 5-ring lactones. Those which are classed as “saccharic acids” can form also a double lactone having two 5-membered rings; but this is not possible with the “mucic acids.”

¹⁰ Derivation of this formula is as follows: Let *k_f* and *k_s* be the velocity constants for the formation and splitting of a lactone, respectively. *K* is the equilibrium constant. The amount of original acid is *a*, and the amount of lactone at time *t* is *x*. Then,

$$\frac{dx}{dt} = k_f(a - x) - k_s x \quad (2)$$

Let *A* equal the fraction changed at equilibrium, then,

$$K = \frac{k_f}{k_s} = \frac{A}{1 - A} \text{ or } A = \frac{K}{K + 1} \quad (3)$$

From equations (2) and (3)

$$\frac{dx}{dt} = k_f \left(a - x \frac{K + 1}{K} \right) = \frac{k_f}{A} (Aa - x) \quad (4)$$

Integrating:

$$k_f t = A \ln \frac{A}{A - \frac{x}{a}} = A \ln \frac{A}{A - X} \quad (5)$$

where *X* is the fraction in the lactone form at time *t*.

The above results on monocarboxylic acid lactones indicate that the nearer a hydroxyl is to a carboxyl group in the same molecule, the less rapidly does it form a lactone (although to a larger extent). In the dicarboxylic acids we should expect the different lactones to be more nearly alike since each hydroxyl is influenced by two carboxyl groups—one on each end of the molecule. The rates of formation and points of equilibrium should be more nearly the same for the different types of lactones than was the case with the monocarboxylic acids. The rates should be slower than those of the monocarboxylic acids.

The results of experiments on dicarboxylic acids indicate that

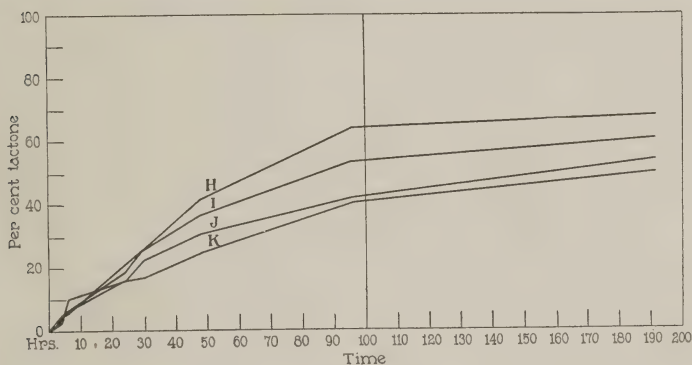


FIG. 3. Lactone formation of dicarboxylic acids at 40°C.

H = Allomucic acid.

I = Mucic acid (in equilibrium with a solid lactone).

J = Saccharic acid.

K = Mannosaccharic acid.

all this is true. The curves of Fig. 3 show that four of these substances are converted into lactones at approximately the same rates and behaving as essentially monomolecular reactions without any sharp breaks in the curves. From this we may conclude either that only a single type of lactone is produced or that two or more lactones are produced, but at nearly the same rates or that the curve is not continued far enough to make a break obvious.

The question of the number of lactones is, however, answered in the case of mucic acid, at least. From Table VII we calculate that at 30 hours 0.0052 mol per liter of lactone is in solution in equilibrium with solid lactone (precipitated). At 48 hours this

increases to 0.0068 mol; at 96 hours, 0.0084 mol; and at 192 hours, there is 0.0094 mol per liter. If there had been but a single type of lactone present this value would be constant. From the first and last concentrations we may calculate that *over 22 per cent of the lactone which is fomed at 192 hours is another type than that which precipitated.* It is probable that more than one type of lactone is produced from the other dicarboxylic acids also.

The data in Fig. 3 were obtained by titration. Since the results are not entirely satisfactory we have not calculated reaction constants, but present the results for their qualitative significance. The experiments on dicarboxylic acids were performed at 40°C. in 0.0125 molar solutions. The higher dissociation constants counterbalance the lower concentration so that the pH at the start was practically the same in the experiments with monocarboxylic acids. The rate of lactone formation of dicarboxylic acids at 40°C. was essentially the same as the 5-ring lactone formation from monocarboxylic acids at 25°C. Hence, under the same conditions, the dicarboxylic acids form lactones at even a slower rate than that at which 5-ring lactone is produced from monocarboxylic acids. From the monocarboxylic acids, formation of 6-ring lactone occurs to 30 per cent or less and 5-ring lactone, to about 75 to 80 per cent. The limit which is approached in the first 200 hours appears to be about 75 per cent (calculated on the basis of a single lactone). These reactions were not carried to completion.

EXPERIMENTAL.

1/160 of a molecular weight (allowing for moisture) of a lactone of each of the acids in Tables III and IV was weighed out and dissolved in 1.2 equivalents of normal NaOH (7.5 cc.). After the lactone was hydrolyzed, 1.2 equivalents (7.5 cc.) of normal HCl were added (the end-point after the addition of 0.2 equivalent was observed with phenolphthalein). The solution (in a volumetric flask) was quickly made up to 25 cc. (making the solution 0.25 molar) with distilled water, and transferred to a polariscope tube. A reading was immediately taken and subsequent readings were taken after various time intervals. 4 dm. tubes were used when the solutions were clear enough and sufficient material was available. Otherwise, shorter tubes were used.

The results are given in Tables III and IV together with the "per cent" of formation, which was calculated from equation (1) as explained above. The rotations of the pure lactones were obtained on separate solutions dissolved in the cold; except substances (F) and (G) in which cases the values of Levene and

TABLE III.

Lactone Formation of Free Monovalent Sugar Acids at 25°C.

(All concentrations were 0.250 molar.) (See Fig. 1.)

Acid.....{	Gulonic (A).		Galactonic (B).		Glucosheptonic (C).		4-Methyl glucosheptonic (E).	
	4 dm.		2 dm.		2 dm.		4 dm.	
Length of tube.....								
Time in hrs.....{	[M] _D	Lac- tone.	[M] _D	Lac- tone.	[M] _D	Lac- tone.	[M] _D	Lac- tone.
	<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>	
0 (pure acid, [M] _A) _D .	-11°	0	-24°	0	-24°	0	-27°	0
1	-5.5°	+6.3	-17.2°	+5.9	-17.8°	+7.4	-17.5°	+6.1
2	-3.5°	+8.6	-17.0°	+6.1	-14.4°	+11.7	-11.4°	+10.1
3	-2.2°	+10.1	-18.8°	+4.5	-12.2°	+14.4	-7.2°	+12.8
4	-1.2°	+11.3	-20.4°	+3.1	-10.4°	+16.6	-4.2°	+14.7
5	-0.7°	+11.8	-21.6°	+2.1	-9.0°	+18.3	-1.4°	+16.5
23	-10.5°	+0.6	-46.8°	-19.8	-14.6°	+11.5	+6.3°	+21.5
24	-11.2°	-0.2	-48.0°	-20.9	-15.4°	+10.5	+6.3°	+21.5
26	-12.9°	-2.2	-49.8°	-22.4	-16.8°	+8.8	+6.3°	+21.5
30	-15.0°	-4.6	-53.0°	-25.2	-18.8°	+6.3	+6.3°	+21.5
48	-25.3°	-17.6	-67.4°	-37.7	-28.0°	-4.9	+6.3°	+21.5
53	-27.1°	-18.5	-70.4°	-40.3	-30.8°	-8.3	+6.3°	+21.5
72	-37.1°	-30.0	-82.4°	-50.8	-40.0°	-19.5	+6.3°	+21.5
96	-45.7°	-39.9	-92.2°	-59.3	-49.6°	-31.2	+6.3°	+21.5
144	-58.0°	-54.0	-102.2°	-68.0	-62.6°	-47.1	+6.3°	+21.5
264	-71.2°	-69.2						
Equilibrium.	-85.0°	-85.0	-112°	-76.5	-88°	-78.0	+6.0°	+21.3
Pure lactone.* [M] _L) _D	-98.0°	-100	-139°	-100°	-106°	-100	+128°	+100
Types of lactones.	+6-ring and -5-ring.*		+6-ring and -5-ring.*		+6-ring and -5-ring.*		+6-ring* only.	
[M] _L) _D - [M] _A) _D								
Change of rotation due to lactone formation.	-87		-115		-82		+155	

* Predominating lactone at equilibrium.

Meyer⁷ were used. The rotations at equilibrium for (A), (B), and (C) were taken from the data in Table V; for (D) from a separate solution of mannonic lactone; for (G) from Levene and Meyer; and for (E) and (F) from the equilibria reached in the experiment.

The velocities of reaction of (A), (B), (C), and (E) were observed also in the presence of 1 equivalent excess of hydrochloric acid—making the hydrogen ion activity 33 times greater—in

TABLE IV.

Lactone Formation of Free Monovalent Sugar Acids at 25°C.

All percentages are positive (+) in this table. (See Fig. 1.)

Acid.....{	Mannonic (D).		2, 3, 5, 6-Tetramethyl mannonic (G).		2, 3, 4, 6-Tetramethyl mannonic (F).	
Molar concentration.....{	0.250		0.185		(0.250)	
Length of tube.....	2 dm.		2 dm.		1 dm.	
Time in hrs	[M] _D	Lactone.	[M] _D	Lactone.	[M] _D	Lactone.
		per cent		per cent		per cent
0 (pure acid, [M] _A) _D .	-0.6°	0	-59.2	0	+41.0°	0
0.083	+2.4°	+3.2	-55.5°	+1.8		
0.17					+43.6°	+1.3
0.25	+9.6°	11.0	-54.2°	2.4	+45.2°	2.0
0.50	+17.0°	19.0	-54.2°	2.4	+48.0°	3.4
0.75	+24.0°	26.6				
1.00	+28.0	31.5	-52.0°	3.4	+52.0°	5.3
1.50	+38.0°	41.7	-50.7°	4.0	+56.0°	8.3
2.00	+44.0°	48.2				
3.50			-47.2°	5.7	+63.2°	10.8
4.00	+55.0°	60.0	-46.0°	6.3	+66.0°	11.7
5.00	+58.0°	63.3	-43.3°	7.5	+75.2°	16.7
24	+62.6°	68.3	-28.5°	14.5	+100°	28.8
30	+64.0°	69.8	-25.7°	15.9	+101°	29.3
48	+66.0°	72.0	-12.2°	22.3	+106°	31.7
96	+73.4°	80.0	+13.5°	34.4	+111°	34.2
144	+77.2°	84.1	+29.8°	42.2	+(115°)	(36.1)
168	+79.0°	86.0	+33.9°	44.1	+114°	35.6
193	+80.4°	87.5	+40.1°	47.0	+114°	35.6
217	+81.4°	88.5	+43.6°	48.7	+114°	35.6
241	+82.6°	90.0	+46.1°	50.0	+114°	35.6
256	+83.4°	90.7	+48.8°	51.2	+114°	35.6
Equilibrium.	+84°	+91.4		(+75)	+114°	+35.6
Pure lactone.*[M] _L) _D	+92	100	+152°	100	+246°	100
Types of lactones.	+6-ring and +5-ring.*		+5-ring* only.		+6-ring* only.	
[M] _L) _D - [M] _A) _D	+92.6°		+211°		+205°	

* Predominating lactone at equilibrium.

the case of (A). The same factor applies approximately to (B), (C), and (E). The results are given in Table V. (See also Fig. 1.)

The lactone formation of a solution of free gulonic acid (A)

TABLE V.

Lactone Formation of Monovalent Sugar Acids at 25°C. in Solutions Containing 1 Equivalent of Free Hydrochloric Acid.

All concentrations were 0.250 molar. All tubes were 2 dm. long. (See Fig. 1.)

Acid.....		Gulonic (A).		Galactonic (B).		Glucoseheptonic (C).		4 Methyl glucoseheptonic (E).	
Time.	(Time) x 33	[M]D	Lactone.	[M]D	Lactone.	[M]D	Lactone.	[M]D	Lactone.
hrs.			per cent		per cent		per cent		per cent
0	0	(-11°)	0	(-24°)	0	(-24°)	0	(-27°)	
1	33	-16.8°	-6.7	-53.0°	-25.2	-20.0°	+4.9	+6.0°	
1.5	50	-25.8°	-17.0	-67.6°	-37.9	-28.0°	-4.9	+6.0°	21.3
2.0	66	-34.8°	-27.4	-78.0°	-47.0	-35.6°	-14.1	+6.0°	21.3
2.5	83	-42.4°	-36.0	-86.0°	-53.9	-42.4°	-22.4	+6.0°	21.3
3.5	115	-54.0°	-49.5	-96.0°	-62.6	-53.6°	-36.1	+6.0°	21.3
4.5	140	-62.0°	-58.7	-102.6°	-68.4	-61.2°	-45.4	+6.0°	21.3
7	232	-74.0°	-72.5	-108.8°	-73.8	-74.6°	-61.7	+6.0°	21.3
24	792	-85.0°	-85.0	-112.0°	-76.5	-88.2°	-78.0	+6.0°	21.3
28	924	-85.0°	-85.0	-112.0°	-76.5	-88.2°	-78.0	+6.0°	21.3
48	1584	-85.0°	-85.0	-112.0°	-76.5	-88.2°	-78.0	+6.0°	21.3
Equilibrium.		-85°	-85	-112°	-76.5	-88°	-78	+6.0°	+21.3
Per cent by titration.			(75)		(76)		(53)		(4)

For types of lactones and rotation changes, see Table III.

TABLE VI.

Lactone Formation of Free Gulonic Acid at 25°C., Measured Both by Optical Rotation and by Volumetric Titration.

Concentration = 0.250 molar. Length of tube = 4 dm.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Time.	Rotation.		Titration.		6-ring lactone. (Column 3 + Column 5.)	6-ring + 5-ring lactones. (Column 6 + Column 5.)
	[M] _D	6-ring - 5-ring lactone.	Volume 0.10 M NaOH.	5-ring lactone.		
hrs.		per cent	cc.	per cent		
0	-11.0°	0	2.50	0	0	0
0.5	-6.0°	+5.7	2.42	3.2	8.9	12.1
1	-4.0°	+8.0	2.39	4.4	12.4	16.8
2	-2.4°	+9.9	2.29	8.4	18.3	26.7
4	-0.8°	+11.7	2.19	12.4	23.1	35.5
6	-1.6°	+10.8	2.12	15.2	26.0	41.2
24	-16.0°	-5.7	1.74	30.4	24.7	55.3
30	-20.4°	-10.8	1.65	34.0	23.2	57.2
48	-31.6°	-23.7	1.45	42.6	18.3	60.3
96	-51.8°	-47.0	1.05	58.0	11.0	69.0
144	-66.0°	-63.2	0.79	68.4	5.2	73.6
See Fig. 2, Curve		I		II	III	IV

was observed both optically and by titration. The latter readings probably correspond closely to the amount of 5-ring lactone present, since most of the 6-ring lactone (and perhaps a little of

TABLE VII.

Lactone Formation of Free Dicarboxylic Sugar Acids at 40°C.

All concentrations = 0.0125 molar. (See Fig. 3.)

Acid...	Saccharic (J).			Mannosaccharic (K).			Mucic (I).				Allomucic (H).		
	V ₁	% _l	ΔV ₂	V ₁	% _l	ΔV ₂	V ₁	% _l	ΔV ₂	% ₂	V ₁	% _l	ΔV ₂
<i>hrs.</i>													
0	2.51	0	0	2.51	0	0	2.50	0	0		2.51	0	0
2	2.49	1.6	0.02	2.48	2.4	(0.11)	2.48	1.6	(0.11)		2.49	1.6	(0.14)
4	2.48	2.4	0.03	2.45	4.8	(0.10)	2.44	4.8	0.06		2.46	4.0	(0.10)
6	2.45	4.8	0.05	2.43	6.4	(0.17)	2.43	5.6	(0.16)		2.47	6.4	(0.17)
24	2.31	16.0	0.24	2.31	16.0	(0.18)	(Precipitate.)				2.28	18.4	0.27
30	2.23	22.4	0.33	2.30	18.8	(0.37)	1.79		0.26	25.4	2.19	25.6	0.41
48	2.13	30.4	0.38	2.20	24.8	0.33	1.48		0.34	37.4	1.99	41.6	0.53
96	1.99	41.6	0.48	2.01	40.0	0.49	1.16		0.42	53.2	1.71	64.0	0.78
192	1.84	53.6	0.66	1.89	49.6	0.69	1.10		0.47	60	1.67	67.2	0.84

V₁ = Volume of 0.10 M NaOH required to neutralize 10 cc. of solution (to phenolphthalein).

%_l = Corresponding per cent of (single) lactone.

ΔV₂ = Difference in titration value resulting from splitting the lactone with excess of NaOH, and back titration with HCl. V₁ + ΔV₂ should equal 2.50 to 2.51 cc.

%₂ = (For mucic acid after precipitation of lactone began) = $100 \frac{\Delta V_2}{V_1 + \Delta V_2}$.

TABLE VIII.

Titration of 0.0250 Molar Gulonic Acid at 25°C.

(0.10 M HCl = pH 1.090. Potential of saturated KCl junction assumed constant.)

pH	$\frac{B}{C}$	$\alpha = \frac{B}{C} + \frac{H}{f_H C}$	pK + log <i>f</i>	log <i>f</i>	pK
4.56	0.900	0.900	3.60	-0.10	3.70
4.21	0.800	0.802	3.61	-0.10	3.71
3.95	0.700	0.705	3.57	-0.10	3.67
3.75	0.600	0.608	3.56	-0.10	3.66
3.24	0.300	0.326	3.56	-0.10	3.66
2.67	0	0.096	(3.64)	-0.10	(3.74)
Best average....			3.58		3.68

$K = 10^{-3.68} = 2.1 \times 10^{-4}$ (corrected for activity).

the 5-ring lactone) appears to be split in the titration. The results are given in Table VI and plotted in Fig. 2.

The dicarboxylic acids were studied by titration. Most of

the results were unsatisfactory and are not presented here. Owing to the extreme insolubility of lactones from the two mucic acids, it was necessary to use a dilute solution (eightieth molar). Even at that concentration the lactone from mucic acid precipitated before 7 per cent had formed. The data for this acid are calculated on the basis of the amount of material left in solution at each point. The amount of free acid was found by titration with NaOH, while the lactone was determined by back titration with HCl after splitting the lactone with NaOH. Since the amount of lactone in solution (in equilibrium with solid) was not constant but increased slightly, it is evident that more than one form was produced. In the case of each of the other acids, the lactone formed by titration with NaOH agreed with that formed by back titration with HCl (since no lactone was precipitated).

The solutions were made from the free dicarboxylic acids, but were treated with 2.2 equivalents of NaOH, to make sure that no lactone was present, and then with 2.2 equivalents of HCl. 10 cc. portions were titrated with 0.1 M NaOH from a burette accurate to 0.01 cc. The results are given in Table VII and are plotted in Fig. 3.

The dissociation constant of gulonic acid was determined from an eightieth molar solution of the salt, to samples of which, varying amounts of acid were added and the pH determined as quickly as possible in order to avoid lactone formation. The observations at the lower pH values are less reliable, due to lactone formation. The results are given in Table VIII. The constant is given by the equation:

$$K = f_H \frac{\alpha}{1 - \alpha} \quad (6)$$

$$\text{where } \alpha = \frac{\frac{H}{f_H} + B}{C} \quad (7)$$

H = Hydrogen ion activity.

$\frac{H}{f_H}$ = " " concentration.

B = Concentration of strong base (NaOH).

f = Activity coefficient of gulonic anion (more accurately the ratio of the activity coefficients of the anion and the undissociated molecule, unless the latter is unity).

f_H = Activity coefficient of hydrogen ion.

We assume at 25°C.:

$$-\log f = 0.30 \sqrt{\Sigma i^{\nu}} \quad (8)$$

where Σi^{ν} is the sum of all the ion concentrations i , each raised to the power ν equal to its valence. In this experiment $0.30 \sqrt{\Sigma i^{\nu}}$ is equal to approximately 0.10 in each case. Therefore:

$$\begin{aligned} \text{pK} &= \text{pH} - \log \frac{\alpha}{1 - \alpha} - \log f \\ &= \text{pH} - \log \frac{\alpha}{1 - \alpha} + 0.10 \end{aligned} \quad (9)$$

Equation (8) is sufficiently accurate for obtaining values of f_H in equation (7).

SUMMARY.

It was found (as predicted from the structural models) that normal monocarboxylic acids derived from hexoses (and also heptoses) are practically identical in the rates of their lactone formation. Likewise, the isomeric dicarboxylic acids are identical with each other in lactone formation. (See Table I.)

The monocarboxylic acids form two lactones simultaneously: one with a 6-membered ring which reaches an equilibrium of 20 to 30 per cent in a few hours; and another with a 5-membered ring which attains an equilibrium of 75 to 80 per cent after several hundred hours. The initial speed of the former reaction is eight times that of the latter. The various acids are all nearly identical.

Certain methylated acids in which only one type of lactone could be produced showed in each case an initial speed and an equilibrium corresponding to the type of ring.

The above results were obtained polarimetrically. In each case the rotation of the lactone was *more dextro* than the acid when the linkage involved a *dextro* carbon atom; and *more levo* when a *levo* atom was involved.

The rates of lactone formation of several dicarboxylic acids, as observed by titration, were essentially identical and bore no relation to the solubility of the various lactones, nor to their ease of isolation. Within the range studied (200 hours) the reactions were essentially monomolecular, having no sharp breaks such as were

found in the curves for the monocarboxylic acids. However, the reactions were not carried to completion. It was not possible to determine which of the four (in some cases, five) possible lactones were forming, but in the case of mucic acid, at least 22 per cent of lactone was produced which was not in the predominating form. This is probably true of the other acids also.

Under the same conditions the formation of lactones from the dicarboxylic acids is somewhat slower than the formation of the lactone with the 5-membered ring from monocarboxylic acids, but appears to approach about the same equilibrium.

The dissociation constant of gulonic acid was found to be $10^{-3.68}$ (corrected for activity).

THE CONFIGURATIONAL RELATIONSHIPS BETWEEN β -HYDROXY ACIDS AND α -HYDROXY ACIDS AND BETWEEN THE LATTER AND SECONDARY ALCOHOLS.

BY P. A. LEVENE AND H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Much progress has been made in recent years in our knowledge of the configurational relationships in the groups of α -hydroxy acids, α -amino acids, and α -halogen acids, and of the configurational interrelationships of the substances of one of these groups to those of the other two groups, as well as of the configurational relationship of these substances to the sugars.

A summary of the work in this direction will appear in another place. Here it suffices to point out that the foundation of our knowledge in this domain was laid by data obtained by purely chemical methods and that subsequently indirect methods were discovered which permitted the establishment of configurational relationships in those cases in which purely chemical methods were not applicable.

But, we possess little accurate knowledge, if any, as to the configurational relationships of those alcohols and hydroxy acids in which the hydroxyl group is present in other than the α position.

In regard to the alcohols, Pickard and Kenyon¹ and, to some extent, Clough² attempted to solve the problem by physical methods. The conclusions of Pickard and Kenyon are at variance with those of Clough and a final decision between the two theories will be reached only by methods of organic chemistry.

No data at all exist regarding the configurational relationship of β -hydroxy and of β substituted acids in general.

The reason that the two groups of substances are discussed

¹ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1914, cv, 2226.

² Clough, G. W., *J. Chem. Soc., Proceedings*, 1913, xxix, 357.

here together is of a practical nature. It is possible, starting out from the β -hydroxy acids to correlate the configurations of the β -hydroxy acids with those of the α -acids on one hand, and then the configuration of each of these with the alcohols. The method of correlating the configurations of the α - and the β -hydroxy acids can be illustrated in the case of β -hydroxybutyric acid, which can be converted into lactic acid by the following set of reactions.



Through this set of reactions, in none of which the asymmetric carbon atom is involved, the configurational relationships of the two groups of acids can be established.

On the other hand, if the amino group in the hydroxylamine is substituted by halogen, the halide may serve as the starting point for a series of alcohols according to the following set of reactions.



In a very evident way $\text{CH}_3\text{CHOHCH}_2\text{Cl}$ can be converted into any one of the higher homologues of ethylmethyl carbinol of the "methyl" series.

On the other hand, the β -hydroxyvaleric acid could easily lead either to α -hydroxybutyric acid or to the homologous secondary alcohols of the "ethyl" series. In fact, the conversion of this acid into ethylmethyl carbinol is even simpler than that of β -hydroxybutyric acid, as may be seen from the following set of reactions.



The higher optically active homologues can thus be prepared in an obvious manner.

It must be admitted, however, that the work is very laborious and time-consuming. It is intended to carry out all the details of the work in this laboratory. The present communication contains the report of only one initial phase of the work, but

even as such, it is important, as it shows that the principal reactions involved in this work are carried out without marked racemization. This phase of the work deals with the conversion of the levo- β -hydroxybutyric acid into levo-1-amino-2-hydroxypropane and of this into the levo-1,2-dihydroxypropane.

For the next step of the work larger quantities of 1, 2-dihydroxypropane will be required and it will be attempted to prepare this by a less complicated method than the above process.

EXPERIMENTAL.

n-Levo-3-Hydroxybutyric Acid.—The procedure for the preparation of this acid was essentially the same as that described by McKenzie.³ An aqueous solution of the inactive acid was heated on the steam bath and neutralized with quinine. The thick syrup was placed in the refrigerator at 5°C. It soon solidified. The solid mass was filtered on a Büchner funnel. The quinine salt thus obtained was fractionally crystallized from water and decomposed with a slight excess of 10 per cent sodium hydroxide. The quinine was removed with chloroform and the aqueous solution concentrated to a small volume. The solution was again extracted with chloroform, and after addition of 10 per cent sulfuric acid equivalent in amount to the alkali previously added, the levo-3-hydroxybutyric acid was extracted with ether in a continuous extraction apparatus. The ethereal extract was dried with anhydrous sodium sulfate and the ether removed under diminished pressure. An aqueous solution, the concentration of which was determined by titration, had the following rotation.

$$[\alpha]_D^{25} = \frac{-1.24^\circ \times 100}{1 \times 5.06} = -24.5^\circ$$

Methyl-n-Levo-3-Hydroxybutyrate.⁴—25 gm. of *n*-levo-3-hydroxybutyric acid were added slowly to 125 cc. of a cold 30 per cent solution of dry hydrogen chloride in methyl alcohol. After standing in the refrigerator at 5°C. for 24 hours, the excess methyl alcohol was distilled off under diminished pressure. The ester

³ McKenzie, A., *J. Chem. Soc.*, 1902, lxxxi, 1402.

⁴ Fischer, E., and Scheibler, H., *Ber. chem. Ges.*, 1909, xlii, 1221.

was dissolved in ether and dried over anhydrous sodium sulfate. After removing the ether, the ester was distilled under diminished pressure. It boiled at 70–72°C., $p = 17$ mm. It had the following rotation without solvent.

$$[\alpha]_D^{20} = \frac{-11.05^\circ \times 1}{0.5 \times 1.058} = -20.9^\circ$$

n-Levo-3-Hydroxybutyrylhydrazide.—This substance was prepared by the procedure employed by Levene and Scheidegger⁵ for the preparation of the inactive 3-hydroxybutyrylhydrazide. After recrystallization from alcohol it melted at 129–130°C.

0.1000 gm. substance: 20.60 cc. N_2 at 24°C. and 756.8 mm.

$C_4H_{10}O_2N_2$. Calculated. N 23.7.

Found. " 23.6.

The rotation in absolute alcohol was

$$[\alpha]_D^{31} = \frac{-0.60^\circ \times 100}{1 \times 2.05} = -29.3^\circ$$

Sym.-n-Dextro-2-Hydroxypropylurea.—10 gm. of levo-3-hydroxybutyrylhydrazide were converted to levo-2-hydroxypropylurea as described by Levene and Scheidegger⁵ for the preparation of the inactive urea derivative. The dilute alcoholic solution, after refluxing, was concentrated to a thick syrup. This syrup had a rotation of $\alpha = +27.35^\circ$ in a 1 dm. tube at 26°C. On drying at a pressure of 0.5 mm., the syrup crystallized readily. It melted at 49–51°C., and analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 9.75 cc. 0.1 N acid.

$C_7H_{16}O_3N_2$. Calculated. N 15.9.

Found. " 13.8.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{25.5} = \frac{+0.37^\circ \times 100}{1 \times 2.00} = +18.5^\circ$$

Levo-2-Hydroxypropylamine.—The procedure for the preparation of this substance was the same as that employed by Levene

⁵ Levene, P. A., and Scheidegger, J., *J. Biol. Chem.*, 1924, lx, 179.

and Scheidegger⁵ for the preparation of the inactive amine. The reaction mixture from 3 gm. of the urea derivative was diluted to 10 cc. with water and had a rotation of $\alpha = -4.35^\circ$ in a 1 dm. tube and 26°C . The acid solution was concentrated to a thick syrup and placed in the refrigerator overnight when it crystallized. It was filtered off and washed with an ice-cold solution of alcohol containing hydrogen chloride (1 part) and ether (3 parts). It was very hygroscopic. It analyzed as follows:

0.0372 gm. substance: 8.44 cc. N_2 gas (Van Slyke) at 28°C . and 750.5 mm.
 $\text{C}_3\text{H}_{10}\text{ONCl}$. Calculated. N 12.55.
 Found. " 12.21.

The rotation of the amine hydrochloride in water was

$$[\alpha]_{\text{D}}^{25} = \frac{-0.50^\circ \times 100}{1 \times 1.6} = -31.2^\circ$$

Another sample, the rotation of which was

$$[\alpha]_{\text{D}}^{25.5} = \frac{-0.60^\circ \times 100}{1 \times 2.45} = -24.4^\circ$$

had the following rotation in a solution of normal sodium hydroxide.

$$[\alpha]_{\text{D}}^{25.5} = \frac{-0.23^\circ \times 100}{1 \times 2.36} = -9.7^\circ$$

Levo-1, 2-Dihydroxypropane.—2.5 gm. of levo-2-hydroxypropylamine hydrochloride were dissolved in 30 cc. of water and 1 equivalent of silver nitrite was added slowly with constant shaking. After standing for 4 hours, slightly more than 1 equivalent of silver nitrite and 1 equivalent of 10 per cent hydrochloric acid were added. After standing at 15°C . overnight the excess silver was removed, the solution filtered, and concentrated under diminished pressure to half its volume. After neutralizing with barium hydroxide, the solution was concentrated to dryness. The residue was made acid to Congo red with hydrochloric acid, extracted with chloroform, and the extract dried with anhydrous sodium sulfate. The chloroform was removed under diminished pressure and the residue dissolved in water. The solution was levorotatory and contained no amino nitrogen.

BLOOD SUGAR TIME CURVES FOLLOWING THE INGESTION OF DIHYDROXYACETONE.

By I. M. RABINOWITCH.

WITH THE ASSISTANCE OF ALTHEA B. FRITH AND ELEANOR V. BAZIN.

(From the Department of Metabolism, the Montreal General Hospital,
Montreal, Canada.)

(Received for publication, June 2, 1925.)

In a preliminary report¹ it was shown that the diabetic is apparently able to tolerate dihydroxyacetone more readily than glucose. In the same subject the character of the blood sugar time curve following dihydroxyacetone ingestion differed from that obtained after glucose. With dihydroxyacetone the maximum increment in the blood sugar was less, and the period of decay, that is the time necessary for the blood sugar to return to its original level, was shorter. In the cases reported it was shown that dihydroxyacetone could be substituted for doses of insulin for the period of observation. Shortly afterwards² an opportunity was afforded to test the use of this substance in a case of diabetes mellitus with severe acidosis. The results obtained were sufficient to stimulate further observations.

At first when dihydroxyacetone was substituted for insulin, an equivalent quantity of other carbohydrates in the diet was deducted from the total allowed. Two other observations were made, though no great significance was attached to them at the time. In one subject, following hydroxyacetone ingestion, the degree of hyperglycemia decreased. In this case and three others, a decreased rate of excretion of sugar in the urine was also noted. In all the subjects large doses of dihydroxyacetone were used; namely, 25 to 100 gm. With the smaller doses (25 gm.), the magnitude of the increments in the blood sugar was such as might be expected in normal persons. The one curve showing a decre-

¹ Rabinowitch, I. M., *Canad. Med. Assn. J.*, 1925, xv, 374.

² Rabinowitch, I. M., *Canad. Med. Assn. J.*, 1925, xv, 520.

ment with no initial increment, belonged to this group. In the case of diabetes with severe acidosis, small doses were used, and here also a fall in the blood sugar was noted. During the period of increment with the smaller doses, no dihydroxyacetone was detected in the blood.

Since no dihydroxyacetone was detected in the blood, the increment in glycemia was attributed to glucose. Two possible causes of this phenomenon suggested themselves. First, during the time

TABLE 1.

Blood Sugar Time Curves Following Ingestion of Dihydroxyacetone.

Determination No.	Before administration.	30 min. after.	60 min. after.	120 min. after.	180 min. after.	240 min. after.	300 min. after.	Remarks.
1	0.095*	0.104	0.082	0.076	0.065			Normal.
2	0.097	0.097	0.092	(?)	0.080			"
3	0.107	0.113	0.098	0.088	0.093			"
4	0.087	0.095	0.076	0.070	0.068			"
5	0.102	0.109	0.078	0.088	0.098			"
6	0.098	0.095	0.111	0.100	0.105			"
7	0.072	0.078	0.071	0.078	0.072			"
8	0.377	0.400	0.400	0.400	0.286	0.307	0.306	Diabetic.
9	0.222	0.232	0.227	(?)	0.202			"
10	0.186	0.200	0.196	0.166	0.153			"
11	0.166	0.175	0.181	0.164	0.138			"
12	0.182	0.208	0.232	0.117				"
13	0.415	0.401	0.400	0.355	0.339			"
14	0.300	0.310	0.270	0.280	0.270			"

* Blood sugar per cent.

the dihydroxyacetone was present in the alkaline intestinal canal, part of it may have been converted into glucose. The ease with which dihydroxyacetone is polymerized in even slightly alkaline media, is recognized. Secondly, assuming that very little polymerization took place in the intestine, when given in larger doses, the increment may have been due to the rate of absorption exceeding the rate of oxidation. An obvious means of partly eliminating both possibilities was to administer dihydroxyacetone in small quantities equal to, or less than, the possible rate of oxidation.

Whether these explanations are correct or not, can, as yet, not be stated definitely. When, however, dihydroxyacetone was

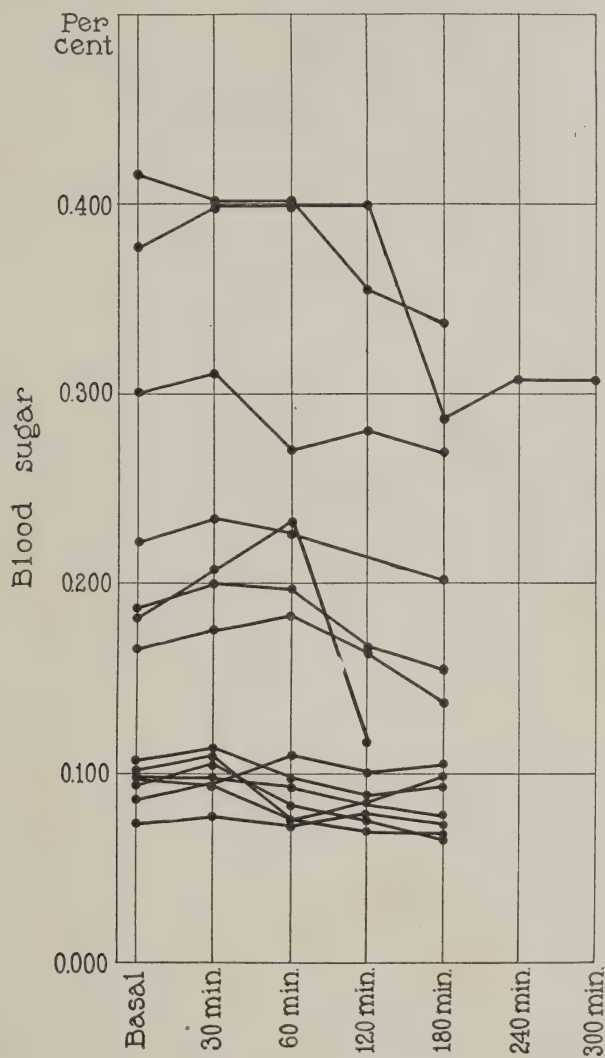


FIG. 1.

given to any subject in a quantity not greater than that corresponding to the determined rate of utilization of carbohydrates

in that individual (determined by respiratory quotient and total metabolism studies), there was only a slight initial increment or none at all. This was followed by a fall in the blood sugar below the level noted prior to administration.

The technical methods employed for blood sugar and dihydroxyacetone determinations, were those previously recorded.¹ Table I shows the combined data obtained in normal and diabetic subjects. These are graphically recorded in Fig. 1.

When dihydroxyacetone is administered by mouth in proper dosage it has an insulin-like action in that it lowers the blood sugar. It is thus not only more readily tolerated than glucose by the diabetic, but actually has some influence on the metabolism of the carbohydrates already present in the body.

The clinical importance of this observation is apparent. Since this was made it has been found possible to substitute dihydroxyacetone for doses of insulin without, as previously, deducting a corresponding quantity of other carbohydrates from the diet. A detailed study of the clinical use of this substance will be published shortly.

THE NITROGEN DISTRIBUTION AND PERCENTAGES OF SOME AMINO ACIDS IN THE MUSCLE OF THE SHRIMP, PENEUS SETIFERUS (L.).

By D. BREESE JONES, OTTO MOELLER,
AND CHARLES E. F. GERSDORFF.

*(From the Protein Investigation Laboratory, Bureau of Chemistry, United
States Department of Agriculture, Washington.)*

(Received for publication, June 17, 1925.)

The amino acid composition of muscular tissue from various sources is recorded in chemical literature. The hydrolysis and complete analysis of the muscle proteins of chicken (1), halibut (2), ox (3), and scallop (4) have been made. Percentages of the diamino acids have also been determined by the Van Slyke method in the muscular tissue of rabbit, chicken, ox, horse, sheep, and pig (5). Similar analyses of various tissues from the same, and from different, animals are also recorded (6, 7). With but one exception, all of these citations refer to work done on the muscle of fish and of animals of higher forms of life.

So far as we are aware, the amino acid composition of the muscle of a crustacean has never been determined. Having available a quantity of fresh shrimp which was procured for studies in progress on the nutritive value of certain types of sea food, it was considered of interest to determine the distribution of nitrogen and percentages of some of the amino acids in shrimp muscle. Such data would throw light on the nutritive value of the proteins of this muscle, and would afford a means of comparing the proteins with those of the scallop, as well as with those of the muscle of animals of the higher forms of life.

The diamino acids were estimated by the Van Slyke method, and tyrosine, tryptophane, and cystine were determined colorimetrically. Aspartic and glutamic acids were isolated and determined gravimetrically. The quantities of the amino acids determined are expressed in percentages of the ash- and moisture-free muscle (Table I). There are also included, for comparison,

the percentages of the same amino acids in scallop muscle as found by Osborne and Jones (4). It must be emphasized, however, that these figures are not strictly comparable. Those given for

TABLE I.
*Percentages of Some Amino Acids in Shrimp Muscle.**

Amino acid.	Shrimp muscle.			Scallop muscle.†
	I	II	Average.	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Cystine‡.....	1.78	1.71	1.75	
“ §.....	1.78		1.78	
Arginine‡.....	10.22	10.25	10.24	7.38
Histidine‡.....	3.78	3.78	3.78	2.02
Lysine‡.....	7.53	7.66	7.60	5.77
Tryptophane¶.....		1.21	1.21	Present.
Tyrosine§.....		4.88	4.88	1.95
Aspartic acid 		6.98	6.98	3.47
Glutamic acid 		15.0	15.0	14.88

* Percentages based on the ash- and moisture-free, extracted muscle.

† Osborne and Jones (3).

‡ Determined by the Van Slyke method.

§ Determined colorimetrically (10).

¶ Determined colorimetrically (12).

|| Determined gravimetrically.

TABLE II.
*Distribution of Nitrogen in Four Groups.**

Form of nitrogen.	Shrimp muscle.			Scallop muscle.†
	I	II	Average.	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Humic N.....	0.24	0.22	0.23	0.40
Amide N.....	1.38	1.36	1.37	1.08
Basic N.....	5.96	5.99	5.98	4.52
Non-basic N.....	9.31	9.23	9.27	11.05
Total.....	16.89	16.80	16.85	17.05

* Results expressed in percentages of the ash- and moisture-free muscle.

† Osborne and Jones (3).

scallop muscle were determined by isolation, and, owing to the more or less inevitable losses on separation and purification of the amino acids, must represent minimal values. On the other hand,

those given for the shrimp muscle, excepting aspartic and glutamic acids, were determined by the Van Slyke and colorimetric methods, so that they represent maximal values. The aspartic and glutamic acids also were isolated by different methods in the two cases. The much higher value found for aspartic acid in the shrimp is doubtless to be accounted for chiefly by the use of a recent method for its separation. Of the amino acids determined, glutamic acid offers the most satisfactory basis for making a fair comparison. In this case, the percentages found are practically identical.

In Table II are given figures for the shrimp muscle, calculated from the data obtained by the Van Slyke method of analysis, showing the distribution of nitrogen in four groups. The corresponding values for scallop muscle, as determined by Osborne and Jones, are also included. The data for the scallop muscle were obtained by the method of Hausmann as modified by Osborne and Harris (8). Inasmuch as the methods by which the figures for the shrimp and the scallop were obtained are very similar, these figures afford a fair basis for comparison.

Preparation of Material.

A quantity of the muscle of fresh shrimp, *Peneus setiferus*, was carefully removed from the shells and suspended in 95 per cent alcohol. After standing overnight, the reddish alcoholic extract was drained off and the muscle was ground in a meat chopper and again suspended in alcohol. The extraction with alcohol was again repeated once or twice until the extracts were nearly colorless. The material was then suspended in absolute alcohol, and finally extracted with ether. The product, thus prepared and air-dried, consisted of a white, very light, coarse powder, and was tasteless and odorless. It contained 12.76 per cent of moisture and yielded 3.90 per cent of ash. Analysis showed it to have the following elementary composition (calculated on an ash- and moisture-free basis).

	<i>per cent</i>
C.....	52.93
H.....	6.33
N.....	16.88
S.....	1.55

Analysis of the Shrimp Muscle by the Van Slyke Method.

Samples of about 3 gm. each of the muscle preparation were hydrolyzed by boiling with 100 cc. of 20 per cent hydrochloric acid for 30 hours. The phosphotungstate precipitate was decomposed by the amyl alcohol-ether method (9). The nitrogen distribution figures are given in Table III. The percentages of the basic amino acids, calculated on the ash- and moisture-free sample, are included in Table I.

TABLE III.

*Distribution of Nitrogen in the Shrimp Muscle as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.5002 gm. protein; 0.4220 gm. nitrogen.†

Sample II, ash- and moisture-free, 2.5002 gm. protein; 0.4220 gm. nitrogen.†

Form of nitrogen.	I	II	I	II	Aver- age.
	gm.	gm.	per cent	per cent	per cent
Amide N.....	0.0345	0.0341	8.18	8.08	8.13
Humin N adsorbed by lime.....	0.0058	0.0051	1.37	1.21	1.29
“ “ in ether-amyl alcohol extract...	0.0001	0.0005	0.02	0.12	0.07
Cystine N.....	0.0052	0.0050	1.23	1.19	1.21
Arginine N.....	0.0822	0.0825	19.48	19.55	19.52
Histidine N.....	0.0256	0.0256	6.07	6.07	6.07
Lysine N.....	0.0361	0.0367	8.55	8.70	8.63
Amino N of filtrate	0.2141	0.2112	50.73	50.04	50.38
Non-amino N of filtrate.....	0.0187	0.0195	4.43	4.63	4.53
Total N regained.....	0.4223	0.4202	100.06	99.59	99.83

* Total N regained corrected for solubility of the bases.

† Nitrogen content of protein, 16.88 per cent.

Cystine, Tyrosine, and Tryptophane.

The cystine and tyrosine determinations were made by the colorimetric method of Folin and Looney (10), and the tryptophane determination by the method of May and Rose (11) with the modifications recently described by the writers (12). The results of these analyses are given in Table I. It is of interest to note that the percentage of cystine found by the colorimetric method was practically the same as that obtained by the Van

Slyke method. We have previously (12) directed attention to the close agreement in many cases between the values found for cystine by these two methods.

Aspartic and Glutamic Acids.

A quantity (50 gm.) of the shrimp muscle equivalent to 41.67 gm. of the ash- and moisture-free material was hydrolyzed by boiling with 200 cc. of 20 per cent hydrochloric acid for 36 hours. The hydrolysate was diluted and the suspended humin filtered off. The diamino acids were removed from the filtrate by means of phosphotungstic acid. After removing the excess of the latter, the colorless solution of monoamino acids was concentrated to a small volume under reduced pressure at a temperature not exceeding 40°C.¹ The solution was then saturated with hydrochloric acid gas. After standing for a few days in a refrigerator, the glutamic acid hydrochloride was removed, dissolved in water, and reprecipitated with hydrochloric acid gas. There were obtained 2.96 gm. of pure glutamic acid hydrochloride.

The filtrate and washings from the glutamic acid hydrochloride were concentrated to a thick sirup, and the calcium salts of the dibasic acids were prepared and separated according to the method of Foreman (13). A small quantity of a difficultly soluble material separated with the calcium salts. This material yielded 0.38 gm. of tyrosine.

The mixture of the free dibasic acids obtained from the salts, after the quantitative removal of calcium, weighed 12.93 gm. By triturating this with glacial acetic acid, 3.65 gm. of a sirupy substance were removed. The undissolved dibasic acids (9.28 gm.) were converted into their copper salts, and 6.0 gm. of copper aspartate were isolated in the characteristic crystalline form. The air-dried copper salt had the following composition.

0.2708 gm. substance:	0.0799 gm. CuO.
0.0676 " " "	required 3.55 cc. of 0.1 N acid.
$C_4H_5O_4NCu \cdot 4\frac{1}{2} H_2O$. Calculated. Cu 23.07, N 7.20.	
Found.	" 23.19, " 7.37.

¹ In all cases the concentrating of solutions containing glutamic acid was done at low temperature and under reduced pressure in order to avoid the formation of pyrrolidone carboxylic acid.

The above yield of copper aspartate corresponds to 2.90 gm. of aspartic acid, or to 6.98 per cent of the ash- and moisture-free muscle.

On concentrating the filtrate from the copper aspartate, there soon separated a pale blue copper salt, consisting of microscopic aggregates and exceedingly difficultly soluble in boiling water. It did not have the usual properties of the copper salt of either aspartic or glutamic acids. After removing the copper with hydrogen sulfide, this salt yielded 1.58 gm. of a crystalline substance, which decomposed with effervescence at 203°C. Its hydrochloride in aqueous solution had a specific rotation of +30.03°. These figures agree with the corresponding ones for glutamic acid. The crystallographic and optical properties of this

TABLE IV.

Free Amino Nitrogen of the Shrimp Muscle Compared with the Lysine Nitrogen.

Total N in 2 cc.	N gas from 2 cc.	Pressure.	Tempera- ture.	Amino N in 2 cc.	Ratio of amino N to total N.	One-half lysine N by Van Slyke method.
mg.	cc.	mm.	°C.	mg.	per cent	per cent
1.264	1.09	767.9	24	0.6147	4.863	4.32

material were identical with those of pure glutamic acid. This glutamic acid fraction was reconverted into its copper salt, which showed the same unusual properties as before. Copper glutamate usually crystallizes in relatively large, deep blue crystals, containing 2.5 molecules of water, and is moderately soluble in water. This difficultly soluble copper glutamate will be further investigated.

There were further obtained from the filtrate of the copper glutamate, after removal of the copper and saturation with hydrochloric acid, 2.87 gm. of glutamic acid hydrochloride. The total glutamic acid found in this analysis amounted to 6.25 gm., equivalent to 15.0 per cent of the ash- and moisture-free muscle hydrolyzed. The glutamic acid hydrochloride included in this percentage consisted of white crystals, which decomposed with effervescence at 197–199°C. and contained 7.64 per cent of nitrogen (theoretical 7.65 per cent).

Free Amino Nitrogen.

Quantities equivalent to 0.75 gm. of the ash- and moisture-free muscle were dissolved in 2.5 cc. of glacial acetic acid plus 1 cc. of distilled water, and the solutions were diluted with water to 100 cc. 2 cc. of each of the solutions in duplicate were used for the determination of total nitrogen and amino nitrogen. The larger reaction bulb of the Van Slyke apparatus was used in combination with the micro gas burette. The results are given in Table IV.

SUMMARY.

The finely ground muscle of fresh shimp, *Peneus setiferus* (L.), was extracted at room temperature with 95 per cent alcohol, and finally with ether. The air-dried extracted muscle consisted of a white, tasteless and odorless, light, coarse powder, having the following percentage composition, calculated on an ash- and moisture-free basis: C 52.93, H 6.33, N 16.88, S 1.55. Analysis of the muscle preparation by the Van Slyke method gave the following results, expressed as percentages of the total nitrogen: amide N 8.13, humin N 1.29, cystine N 1.21, arginine N 19.52, histidine N 6.07, lysine N 8.63. These figures, calculated as percentages of the corresponding amino acids in the ash- and moisture-free muscle, gave the following values: cystine 1.75, arginine 10.24, histidine 3.78, lysine 7.60. The following percentages of amino acids were obtained by colorimetric methods: cystine 1.78, tryptophane 1.21, tyrosine 4.88. Aspartic acid (6.98 per cent) and glutamic acid (15.0 per cent) were determined gravimetrically. The amino acid composition of shrimp muscle is compared with that recorded for scallop muscle.

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IRON IN NUTRITION.

I. NUTRITIONAL ANEMIA ON WHOLE MILK DIETS AND THE UTILIZATION OF INORGANIC IRON IN HEMOGLOBIN BUILDING.*

BY E. B. HART, H. STEENBOCK, C. A. ELVEHJEM, AND J. WADDELL.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

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Von Bunge was the first to show (1) that milk is very low in iron. He also showed that mammalian young (the guinea pig excepted) are born with extra stores of iron in the spleen and liver and that these reserves become sources of iron for hemoglobin building during the early periods of life when milk is the sole article of the diet.

Abderhalden demonstrated (2) that if the animal was kept for a prolonged period of time on a diet consisting only of milk, anemia would result with a marked decrease in the hemoglobin content of the blood. He further demonstrated that the addition of inorganic iron to the milk diet did not result in an increase in the hemoglobin, although he seemed to find some favorable effect upon the growth of the animal (3). Since the time of these experiments by Abderhalden, it has been assumed that inorganic iron cannot take part in hemoglobin building although Abderhalden himself held no such view. In his well known text on physiological chemistry he states:

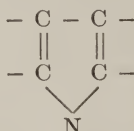
"Enough has been said to show that the formation of hemoglobin does not solve the question as to the part that iron plays in its formation. The kernel of the whole question has not yet been attacked. We cannot hope for a solution of the problem until we understand clearly the formation of hematin. The mere fact that the addition of iron to nutriment poor in iron

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does not have any distinct influence upon the formation of hemoglobin in no way speaks against the participation of inorganic iron in the synthesis of hemoglobin in the case of normal nutrition, but it indicates that other building material is wanting as well as the iron."

That same view has again been expressed by Robertson in his text on "The principles of biochemistry" when he says:

"There is every reason for supposing that the pyrrole group



cannot be synthesized by animals but must be obtained by them pre-formed; that is to say from the tissues of plants or from the tissues of animals which acquired it from plants. This pyrrole grouping is contained in small amounts in the majority of proteins and it forms a very important component of chlorophyll, the green coloring matter of plants, which as we shall see is very closely related chemically to hemoglobin. It is not improbable, therefore, that inorganic iron salts added to an exclusive milk diet are not utilized for building up hemoglobin simply for the reason that other component parts of the hemoglobin molecule as essential as iron itself are either lacking altogether in the milk diet or present therein in insufficient amount to subserve the needs of the blood forming tissues and those of the other tissues of the body as well."

It is unfortunate that in several recent investigations with the rat the original fact observed by Abderhalden that inorganic iron added to a milk diet of some animals will not improve the ration in respect to its capacity to build hemoglobin has not been given the consideration it deserves (4, 5). In these investigations by Mattill and associates, Anderegg and Nelson, and Sure, no data have been presented in reference to the normality or abnormality of the blood, especially in respect to its content of erythrocytes and hemoglobin.

In the work of the aforementioned investigators divers natural materials have been added to the diet with great improvement in the conditions of the rat, especially in respect to its capacity to reproduce. The conclusion that they reached was that vitamin E was deficient in the milk and was introduced by the inclusion of various materials such as wheat embryo and green lettuce leaves. It is, however, true that in most of the recent

investigations on the nutritive properties of milk an exclusive milk diet has not been used, but generally one in which a whole milk powder constituted 50 per cent or more of the ration and of which the remaining portion consisted of lard, corn-starch, and a salt mixture. This was the ration used by Mattill and Stone. The ration used by Anderegg and Nelson contained whole milk powder varying from 5 to 99.8 per cent. When 99.8 per cent was used the remainder of the ration was a citrate of iron. Where the lower levels of milk powder were used, casein, agar-agar, starch, or dextrin constituted the rest. It is not to be expected, on the basis of Abderhalden's work, that an animal receiving a ration of 99.8 per cent of whole milk powder fortified with 0.2 per cent of citrate of iron will be able to build or maintain a blood stream normal in respect to its hemoglobin content. Admitting this to be a fact, we may well ask the question: Should we expect normal rates of reproduction from animals suffering from anemia? This immediately brings up the matter of the availability of inorganic iron as compared with iron in organic combination. Of course, citrate of iron is no more "organic" so far as its relation to hemoglobin building is concerned than is the oxide of iron. It has seemed to us, for some time, that the whole problem of what was involved in normal hemoglobin building needed intensive study, involving the use of inorganic iron as well as the use of complex organic iron compounds; and that in addition there should be studied the rôle in this problem of simple and complex structures such as chlorophyll, which is entirely free from iron. The relation that these factors would have to the recent studies on the nutritive properties of milk mentioned above; to vitamin E itself; and to the large practical problem of the relation of diet to an anemia of suckling pigs observed in England by McGowan and Crichton (6) and very probably related to a similar trouble observed in this country in spring born litters and known as "thumps" is worthy of intensive consideration.

EXPERIMENTAL.

In this paper data are presented which support the original hypothesis of Abderhalden; namely, that hemoglobin can be built from inorganic iron in the diet only when that diet is accom-

panied by certain organic structures. And, further, that milk is not only very low in iron, as is well known, but that it is also low in the organic complex or complexes that make possible the building up of hemoglobin by the organism in the presence of inorganic iron.

For our first experiments rabbits were used. They are easy to bleed from the ear and are very sensitive to a nutritional anemia induced by whole milk feeding. On such a diet an initial hemoglobin content of 85 to 95 per cent and an erythrocyte content of 7 to 8 million per c.mm. will be reduced to 50 to 60 per cent and to 3 to 4.5 million in the course of 5 to 6 weeks. *Yet at the same time growth of the animals continues at an approximately normal rate.* Placed on the liquid whole milk diet at an initial weight of 400 to 500 gm. they will reach weights of 1000 to 1200 gm. in 6 weeks although the blood stream is progressively deteriorating. These increments in live weight, however, will not be continued indefinitely as anemia progresses. When the hemoglobin content of the blood reaches the lower levels of 50 to 55 per cent, growth will cease and death supervene. The remarkable fact is the apparent well being and growth of the animal for such a considerable period of time although the blood stream is abnormal.

In our work we took the rabbits at weaning time (4 to 5 weeks old and 300 to 500 gm. in weight) and placed them in suitable cages on $\frac{1}{2}$ inch mesh screen bottoms. In practically all experimental work with small animals this procedure is now followed in this laboratory (7). It prevents consumption of feces and is highly sanitary. Liquid cow's milk (whole) plus sodium citrate was the only food allowed as the basal ration. The sodium citrate was added at the rate of 3 gm. per liter to prevent the formation of large curds in the stomach and reduce the possibility of gastritis. Very few animals suffered from gastrointestinal disturbances on the diet of whole milk alone, but it nevertheless was a regular procedure in our work to incorporate the sodium citrate, at least for the first 4 weeks of the experimental period. The milk used was from cows that were housed all the time and received no fresh green plant tissue. Whether the diet of the cow would influence the milk in respect to its capacity to build hemoglobin we do not know at present.

Hemoglobin was estimated by the Fleischl hemometer, modified by Miescher. In later work we have also used the Dare hemoglobinometer. The erythrocytes were determined in a standard Thoma-Zeiss hemocytometer.

The recovery type of experiment was the one followed in our later work although in some of the earlier experiments the method of preventing anemia by incorporating various materials with the diet at the time of the initiation of the experiment was followed.

Table I shows a few of our earlier results where the method of prevention rather than that of cure was followed. In these earlier experiments iron only (Fe_2O_3), or fresh cabbage, or fresh cabbage plus iron (Fe_2O_3) was used as a supplement to the milk diet. 50 gm. of cabbage were allowed daily. This amount of cabbage introduced 0.0002 gm. of Fe, as determined by a method which will be published later.

On milk plus sodium citrate the animals invariably came down with anemia.

When cabbage was fed as a supplement at a level of 50 gm. daily per individual, the animals often escaped anemia or were very slow in coming down. When iron (0.015 gm. per individual daily) was fed in addition to the milk-cabbage ration, the blood stream remained normal and there was never any tendency toward an anemia. When iron only (0.015 gm. of Fe_2O_3 per individual daily) was added to the milk diet at the initiation of the experiment, anemia developed in most cases; although the animals were slower in coming down than on milk only. Occasionally an animal would not become anemic on the basal ration supplemented with iron, indicating a possible reserve of hemoglobin-building material in the body or a very efficient use of the small amounts of such material that may be contained in the milk.

We soon learned that we could dry the cabbage at 65°C . for 24 hours and still have it effective as a preventative of anemia when fed with inorganic iron. Data showing results with this material are also incorporated in Table I. It was fed at a level of 5 gm., equivalent to 50 gm. of fresh cabbage per individual per day.

In the administration of the fresh unextracted cabbage a small amount of iron was also given and it became clear that this type of an experiment would not conclusively answer the

question: Are we introducing some organic complex containing iron in traces which can be directly used in hemoglobin building or can the organic complex be iron-free and in the presence of inorganic iron be utilized in hematin formation? Consequently we resorted to the use of certain plant extracts.

TABLE I.

Record of Hemoglobin and Erythrocyte Contents of Blood as Modified by Administration of Cabbage and Iron Oxide.

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythrocytes.	Weight.
			per cent	million per c. mm.	gm.
1	Jan. 6	Whole milk only + sodium citrate.	83	7,175,000	440
	Feb. 17	" "	59	4,200,000	1000
2	Jan. 6	" "	93	8,300,000	615
	Feb. 17	" "	70	5,400,000	740
3	Jan. 6	Whole milk + 50 gm. fresh cabbage daily.	89	7,270,000	475
	Feb. 17	" "	98	7,650,000	1320
4	Jan. 6	" "	94	6,650,000	490
	Feb. 17	" "	92	6,450,000	1440
5	Jan. 6	Whole milk + sodium citrate + 15 mg. Fe_2O_3 daily.	104	7,550,000	510
	Feb. 17	" "	72	5,450,000	1240
6	Jan. 6	" "	117	9,850,000	620
	Feb. 17	" "	96	8,800,000	1240
7	Jan. 6	Whole milk + 15 mg. Fe_2O_3 + 50 gm. cabbage daily.	85	7,480,000	610
	Feb. 17	" "	96	7,300,000	1320
8	Jan. 6	" "	83	6,850,000	555
	Feb. 17	" "	89	6,525,000	1360
9	Jan. 6	Whole milk + 15 mg. Fe_2O_3 + 5 gm. dried cabbage daily.	93	7,150,000	475
	Feb. 17	" "	96	6,500,000	1330
10	Jan. 6	" "	98	7,000,000	630
	Feb. 17	" "	94	6,025,000	1260

In Table II is given the detailed record of a rabbit started on milk plus sodium citrate only. The record is a typical one and depicts the development of anemia; the failure to restore nor-

mality of the blood stream by ferric oxide administration (this administration was continued for 2 weeks); and the final success in the correction of the anemia when in addition to the ferric oxide there was administered the alcoholic extract of dried cabbage equivalent to 50 gm. of fresh cabbage daily. This table illustrates very clearly the general situation that occurs. With an anemia induced by a milk diet only, the administration of inorganic iron will not restore the blood stream to a normal condition; but if in addition to the inorganic iron certain organic structures are added to the diet, recovery is rapid and complete.

TABLE II.

Record of Hemoglobin and Erythrocyte Contents of Blood. Restoration to Normal with Alcoholic Extract of Cabbage.

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythrocytes.	Weight.
			per cent	million per c. mm.	gm.
75	Jan. 6	Whole milk + sodium citrate.	83	7,175,000	440
	" 13	" " + " "	75	5,775,000	615
	" 21	" " + " "	73	4,375,000	790
	" 29	" " + " "	76	4,000,000	850
	Feb. 4	" " + " "	66	4,200,000	840
	" 11	" " + " "	68	4,425,000	1000
	" 17	15 mg. Fe_2O_3 added daily.	59	4,200,000	940
	" 24	15 " " " "	59	3,925,000	870
	Mar. 2	25 mg. alcoholic extract dried cabbage equivalent to 50 gm. fresh cabbage + Fe_2O_3 .	64	3,450,000	
	" 9	" "	68	4,775,000	970
	" 16	" "	76	5,450,000	1050
	" 23	" "	79	5,450,000	
	" 27	" "	81	5,950,000	

In our later experiments the recovery type of experiment has been employed. When the animals showed a hemoglobin content of 50 to 60 per cent and at the same time the erythrocytes were down to 3 to 4.5 million, the particular material to be tested was added to the diet. This was done by triturating the material with a small quantity of milk in a mortar. Material prepared in this manner was readily consumed in most cases.

Further experiments were made to determine if extracts of desiccated cabbage would be as potent as the unextracted plant

tissue in restoring an anemic blood stream to a normal condition, particularly in the presence of inorganic iron. In this respect the experiments were completely successful. The extract was prepared from desiccated cabbage by extracting it for 24 hours at room temperature with 95 per cent alcohol. The alcoholic solution was filtered off and evaporated before a fan at a temperature of about 40°C. A syrupy mass was obtained which was found practically *free from iron* as estimated by our method which will accurately determine as small an amount as 0.0001 gm. The determination of iron by the method in use is finally dependent upon the development of a color with potassium thiocyanate. It took 1 gm. of the extract or 40 times the daily administration to show a faint color with thiocyanate.

This extract was fed at a daily level equivalent to 50 gm. of fresh cabbage and appeared as potent a carrier of the organic complex concerned in hematin building as the unextracted cabbage.

Similar experiments have now been carried out with an alcoholic extract of yellow corn-meal, giving results similar to those obtained with desiccated cabbage. Evidently these two different types of plant material carry certain substances soluble in alcohol, and free from iron, which are intimately concerned in hemoglobin formation. Whatever their nature, they appear to be low in amount or absent from whole milk. See Tables III and IV for the records of these data.

Since it has been suggested that the pyrrole nucleus may be concerned in hematin building, we naturally turned to chlorophyll itself. It is, of course, true that the pyrrole nucleus is contained in the proteins of milk, but apparently from free proline alone hematin formation in the animal cannot take place. Direct experiments to determine this point are in progress. It is entirely possible, however, that the organic substance concerned here is much more complex than a single pyrrole molecule, if it is a pyrrole structure at all; for example, it may consist of a multiple of these molecules in definite union such as has been projected for the structure of both chlorophyll and hematin. The possibilities are many and very inviting and will be studied as time permits.

Chlorophyll was prepared by the well known method of Will-

TABLE III.

Record of Hemoglobin and Erythrocyte Contents of Blood. Restoration to Normal with Alcoholic Extract of Cabbage.

Rabbit No.	Date.	Diet.	Hemo-globin.	Erythrocytes.	Weight.
			per cent	million per c. mm.	gm.
27	Mar. 16	Whole milk + sodium citrate.			260
	" 28	" " + " "	79	6,275,000	330
	Apr. 4	" " + " "	66	3,775,000	410
	" 8	25 mg. alcoholic extract cabbage + 15 mg. Fe ₂ O ₃ .			
	" 15	" "	83	6,550,000	450
	" 22	" "	81	5,950,000	530
12	Nov. 7	Whole milk + sodium citrate.	81	5,500,000	430
	" 18	" " + " "	69	5,400,000	550
	Dec. 3	" " + " "	69	4,800,000	660
	" 15	" " + " "	77	6,050,000	765
	" 22	" " + " "	70	4,975,000	850
	Jan. 3	" " + " "	68	4,725,000	920
	" 15	" " + " "	53	3,500,000	1070
	" 18	25 mg. alcoholic extract cabbage + 15 mg. Fe ₂ O ₃ .			
	" 27	" "	75	5,175,000	1100
	Feb. 4	" "	89	5,250,000	1200
	" 16	" "	89	5,375,000	1320

TABLE IV.

Record of Hemoglobin and Erythrocyte Contents of Blood. Restoration to Normal with Alcoholic Extract of Corn-Meal.

Rabbit No.	Date.	Diet.	Hemo-globin.	Erythrocytes.	Weight.
			per cent	million per c. mm.	gm.
33	Apr. 15	Whole milk + sodium citrate.			
	" 27	" " + " "	59	3,425,000	610
	" 28	25 mg. alcoholic extract corn-meal + 15 mg. Fe ₂ O ₃ .			740
	May 4	" "	68	3,700,000	820
	" 8	" "	77	6,950,000	920
29	Mar. 30	Whole milk + sodium citrate.	75	6,700,000	320
	Apr. 10	" " + " "	81	5,875,000	380
	" 25	" " + " "	47	3,750,000	470
	" 25	25 mg. alcoholic extract corn-meal + 15 mg. Fe ₂ O ₃ .			
	" 30	" "	77	5,950,000	710
	May 11	" "	64	5,700,000	800
	" 13	" "	73	5,600,000	920

stätter from tomato leaves desiccated in the dark and before a fan. The ground dried material was extracted on a Büchner funnel with 80 per cent commercial acetone, allowing the solvent to pass through the powder once and sucking dry after each addition. The acetone solution was added in two separate portions to an equal quantity of petroleum ether, and with each portion, water was added to constitute one-eighth the volume of the petroleum ether. The mixture was gently rotated and mixed to transfer the chlorophyll, xanthophyll, and carotins to the petroleum ether layer. The watery acetone was run off each time and the petroleum ether further washed with two additions of 80 per cent of acetone which was also run off. The trace of acetone remaining in the petroleum ether was removed by washing four times with water. The petroleum ether solution was next washed with three successive additions of 80 per cent of methyl alcohol which removed the xanthophyll. The remaining petroleum ether solution was washed again with four additions of water to remove the last traces of acetone and methyl alcohol. At this stage the chlorophyll was precipitated as a fine suspension in the petroleum ether. This suspension was shaken with dry sodium sulfate and talcum powder and filtered on a Büchner funnel through a layer of talc. The chlorophyll on the Büchner funnel was dissolved in anhydrous, alcohol-free ether, concentrated in the drying room, and finally dried in a desiccator under diminished pressure. *The preparation was free from iron* as determined by our method. No further determinations of chemical or physical constants were made to establish its degree of purity.

This chlorophyll was fed at the level of 25 mg. daily per individual plus the inorganic iron and the whole milk. The results show that the chlorophyll functioned in the same way as fresh cabbage, as the alcoholic extract of dried fresh cabbage, or as the alcoholic extract of corn-meal, in correcting the anemic condition induced by whole milk only. We presume that every phytochemist will admit that there are chlorophyll precursors in such plant tissues as etiolated cabbage leaves or corn-meal, but at present there is no evidence as to their nature. Table V gives a part of the records secured with chlorophyll. In the work so far done, about 75 rabbits have been used, but only a small percentage of the records are here presented.

Are These Complexes of Plant Origin and Concerned in Hematin Building in the Animal Related to Any of the Known Vitamins?

From the fact that nutritional anemia is produced on a whole milk diet which is well supplied with vitamin A, it is evident

TABLE V.

Record of Hemoglobin and Erythrocyte Contents of Blood. Restoration to Normal with Addition of Chlorophyll.

Rabbit No.	Date.	Diet.	Hemo- globin.	Erythrocytes.	Weight.
			<i>per cent</i>	<i>million per c. mm.</i>	
15	Mar. 16	Whole milk only + sodium citrate.			
	" 26	" "	73	7,000,000	210
	Apr. 4	" "	57	4,975,000	325
	" 6	25 mg. chlorophyll + 15 mg. Fe ₂ O ₃ .			370
	" 15	" "	81	3,625,000	370
	" 22	" "	68	2,600,000	390
	" 28	" "	77	3,850,000	360
	May 6	" "	85	6,725,000	420
14	Mar. 16	Whole milk only + sodium citrate.			
	" 26	" "	79	6,625,000	170
	Apr. 1	" "	81	6,425,000	250
	" 10	" "	79	5,575,000	310
	" 17	" "	66	4,325,000	340
	" 18	25 mg. chlorophyll + 15 mg. Fe ₂ O ₃ .			400
	" 22	" "	53	3,500,000	460
	" 29	" "	81	7,050,000	500
	May 6	" "	83	6,425,000	560
34	Apr. 15	Whole milk + sodium citrate.			680
	" 27	" " + " "	62	5,375,000	830
	May 1	" " + " "	62	3,225,000	850
	" 2	25 mg. chlorophyll + 15 mg. Fe ₂ O ₃ .			830
	" 8	" "	77	5,050,000	
	" 13	" "	81	5,050,000	830

that this compound can be dismissed as being concerned in the problem. The same statement can be made with respect to

vitamin B. Whole milk is not classed as a material rich in vitamin B, but there were no symptoms indicating such a deficiency. With respect to vitamin C, there is also no reason to believe that it is concerned in this problem. The rabbit is not sensitive to scurvy¹ and if it were, the amount of milk consumed would have fully protected it. Consumption of 120 to 150 cc. of milk daily was the record for these rabbits and half of that amount of winter-produced milk will fully protect a guinea pig of 300 to 400 gm. from scurvy. Further confirmation of the non-relation of vitamin C to this problem is found in the fact that nutritional anemia¹ is readily induced in chicks on whole milk diets. This animal is certainly not dependent on preformed vitamin C in the diet.

TABLE VI.

Record of Hemoglobin and Erythrocyte Contents of Blood of Normal Rats 5 to 7 Months Old and of Rats Kept 6 to 7 Months on a Synthetic Diet.

Rat No.	Stock ration.		Rat No.	Synthetic ration.	
	Hemoglobin.	Erythrocytes.		Hemoglobin.	Erythrocytes.
	<i>per cent</i>	<i>million per c. mm.</i>		<i>per cent</i>	<i>million per c. mm.</i>
1	145	9,950,000	1	96	10,050,000
2	153	10,700,000	2	117	7,600,000
3	132	9,850,000	3	117	8,450,000
4	128	9,500,000	4	119	6,300,000
5	128	9,700,000	5	134	7,650,000
6	128	9,600,000	6	130	9,450,000
7	128	10,550,000	7	142	11,800,000

As to vitamin D, that also can be dismissed. Anemias on a milk diet were as readily induced when the animal was subjected to ultra-violet irradiation as without.

With respect to the relation of vitamin E to our problem we are not so clear. In the publications of Evans and Bishop (8, 9) or of Sure (5) nothing has been said as to the normality or the abnormality of the blood stream of their animals (rats). Reproduction was reestablished by the addition of just such natural materials as have cured the nutritional anemia described in this paper. It is, of course, readily admitted that vitamin E and the complexes concerned in nutritional anemia induced by a milk

¹ Unpublished data.

diet may be different, but definite proof of this fact will be necessary. In some preliminary work with rats kept for 6 to 7 months on a synthetic diet consisting of casein 18, salt mixture 4, yeast 6, agar-agar 2, dextrin 70, and cod liver oil 2, the hemoglobin and erythrocyte contents of the blood were determined. These data are given in Table VI. In addition there are given the hemoglobin and erythrocyte contents of the blood of rats kept on stock rations and presumably normal.

The data are too limited to decide definitely that these rats are or are not anemic on synthetic diets. From the data there is a suspicion that some of the animals on the synthetic rations were not normal in respect to the hemoglobin content of the blood. On the other hand, some of them on the synthetic diet appeared to have a blood stream just as rich in hemoglobin as those receiving the natural food mixture. Possibly all of the figures are within the range of variation.

In one of their publications (9) Evans and Bishop raise the question as to whether they are dealing with chlorophyll or other plant pigments in restoring lost fertility in rats on synthetic diets by the use of green leaves, etc. With the use of a commercial preparation of chlorophyll—Merck's Phyllosan—they obtained results which are interpreted by them as excluding chlorophyll as having a rôle in their problem.

Should further investigations absolutely exclude vitamin E as a factor concerned in hematin building, then it appears probable that in addition to the protein mixture of milk and the known vitamins there must occur, preformed in the diet, certain complexes needed for hematin formation.

SUMMARY.

1. Rabbits limited to a whole milk (cow's)-sodium citrate diet develop a nutritional anemia characterized by low hemoglobin and erythrocyte contents of the blood.

2. Inorganic iron (Fe_2O_3) added to the basal ration will not *per se* correct this anemia.

3. Inorganic iron in the presence of fresh cabbage or an alcoholic extract of desiccated cabbage or an alcoholic extract of yellow corn-meal will prevent or cure such an anemia. These extracts are free from iron.

4. Chlorophyll, free from iron, will also, in the presence of added inorganic iron, correct such an anemia as is induced by a whole milk-sodium citrate diet. The relation of these substances to vitamin E and to certain recent investigations on the nutritive properties of milk are discussed.

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SOME NITROGENOUS CONSTITUENTS OF THE JUICE OF THE ALFALFA PLANT.

IV. THE BETAININE FRACTION.*

By HUBERT BRADFORD VICKERY.

*(From the Laboratory of the Connecticut Agricultural Experiment Station,
New Haven.)*

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Quaternary bases are widely distributed in plants. The simpler members of this group, in particular choline and the betaines, have received much attention from Schulze and his collaborators and from Stanêk (1). Schulze and Trier (2) state that there can be no doubt that these compounds occur in a larger variety of plants than do the alkaloids, but that the betaines are less widely distributed than choline. This base is always present in plant extracts. A surprisingly large proportion of the nitrogen of the juice obtained from the leafy parts of the alfalfa plant has been found to occur as simple quaternary bases. In this paper are reported the results of an examination of the betaine fraction of the juice of the alfalfa plant, prepared essentially in the manner already described in papers from this laboratory (3). The method of fractionation employed was a slight modification of that reported in a recent paper (4).

In previous attempts to obtain fractions from "alfalfa filtrate" representing definite groups of substances, lead acetate was employed as a preliminary precipitant. It has been found that this reagent precipitates complexes which contain considerable amounts of nitrogenous bases as well as amino acids.¹ Conse-

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¹ Unpublished data.

quently, instead of this reagent, barium hydroxide followed by an equal volume of alcohol was used to remove precipitates which probably contain polybasic organic acids as well as any free aspartic acid present in the juice. The examination of the material obtained by this precipitation will be reported later.

After the removal of reagents, the procedure was as described in a previous paper (4); *viz.*, removal of amino acids, amides, purines, and basic amino acids, by means of Neuberg's reagent (5) (mercuric acetate, sodium carbonate, and alcohol), and precipitation of the quaternary bases of the betaine fraction by means of phosphotungstic acid.

The filtrate from the Neuberg reagent precipitate contained 14.32 per cent of the total nitrogen, but only 2.2 per cent of the amino nitrogen of the alfalfa filtrate. Thus the removal of substances containing amino groups had been very complete. The betaine fraction obtained from it by means of phosphotungstic acid contained 8.48 per cent of the nitrogen of the alfalfa filtrate, showing that quaternary bases form an important part of the simpler nitrogenous constituents of alfalfa juice.

As much stachydrine hydrochloride as possible was removed by crystallization from water and alcohol. The final mother liquor was precipitated in aqueous solution with mercuric chloride. From this precipitate a further small quantity of stachydrine hydrochloride was obtained.

A small amount of trimethylamine was found in the filtrate from the mercuric chloride precipitate. Whether or not this base is a normal constituent of alfalfa or had been produced by secondary decomposition is as yet undetermined. It has long been known as a constituent of certain plants; *e.g.*, *Chenopodium Vulvaria* and *Cratægus Oxyacantha* (6).

The mother liquor of the stachydrine hydrochloride, obtained from the mercuric chloride precipitate, was first precipitated at alkaline and then at acid reaction with Staněk's periodide reagent. The first precipitate should contain the choline and the second the stachydrine. By far the greater part of the nitrogen of the solution was found in the choline precipitate and approximately 70 per cent of this nitrogen was subsequently found, by fractional crystallization of the chloroplatinates, to occur as stachydrine. This experience is similar to that of Schulze and Trier

(7), who likewise found that stachydrine is precipitated along with choline by Staněk's reagent.² By a repetition of the precipitation with this reagent, however, choline was readily obtained.

The precipitate obtained with Staněk's reagent in acid solution should contain stachydrine and any other of the weaker bases of the betaine group which may be present in alfalfa. A small amount of stachydrine was isolated as picrate, and from the mother liquor a chloroplatinate was obtained which appeared to be that of betaine. The amount was too small to permit of certainty in the identification, since only 0.1823 gm. of the chloroplatinate was isolated. Inasmuch as betaine hydrochloride is insoluble in strong alcohol, it is possible that a part of this base had separated with the considerable quantity of sodium chloride previously removed from this solution.

Tables I and II show the yields obtained from the betaine fraction of alfalfa filtrate.

The main betaine fraction obtained from 23.96 kilos of fresh alfalfa contained 4.674 gm. of nitrogen. Of this, 3.359 gm. or 71.9 per cent belongs to stachydrine and 0.321 gm. or 6.86 per cent to choline. Trimethylamine and betaine together make up another 0.0996 gm. or 2.1 per cent. Thus nearly 81 per cent of the nitrogen of this fraction is accounted for as definite substances. How much of the remaining 19 per cent of nitrogen should be assigned to betaine remains to be determined. A part, also, doubtless consists of residues of one or more of the other basic substances.

Stachydrine has been previously obtained from the alfalfa plant by Steenbock (8), as well as in this laboratory (9, 10). The present yield of 0.624 gm. per gm. of nitrogen of the alfalfa filtrate employed, is somewhat larger than that heretofore obtained from hydrolyzed alfalfa filtrate (0.525 gm.), and much larger than the previous yield from unhydrolyzed material

² To test the precipitation of stachydrine in alkaline solution by Staněk's reagent, 0.207 gm. of stachydrine hydrochloride in 30 cc. of water was neutralized with NaOH and 1 gm. of NaHCO₃ added. On adding 5 cc. of Staněk's reagent a precipitate immediately formed, most of which soon became crystalline. This was filtered off after standing 48 hours. Further addition of Staněk's reagent threw out a green oily precipitate. The precipitation of stachydrine at alkaline reaction by this reagent is, therefore, incomplete, and the behavior above recorded is to be expected.

TABLE I.

Distribution of Stachydrine in the Various Fractions of the Main Betaine Precipitate.

	Free base.
	<i>gm.</i>
Direct crystallization of HCl salt.	17.197
From HgCl ₂ precipitate.	2.706
“ periodide precipitate in alkaline solution, “choline fraction”	12.92
From periodide precipitate in acid solution, “stachydrine fraction”	1.60
Total.	34.423

TABLE II.

Yields of Stachydrine, Choline, Trimethylamine, and Betaine Obtained from 23.96 Kilos of Fresh Alfalfa Plant.

Substance.	Amount.	N	Fresh plant.	Dry plant.	N as N of plant.	N as N of juice.	Weight of substance per gm. N in juice.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>gm.</i>
Stachydrine.	34.423	3.359	0.144	0.785	1.51	6.09	0.624
Choline.	2.767	0.321	0.0115	0.063	0.14	0.582	0.0502
Trimethylamine.	0.3046	0.0723	0.0013	0.0069	0.033	0.131	0.0055
Betaine.	0.228	0.0273	0.00095	0.0052	0.0123	0.049	0.0041

TABLE III.

Analysis of 2500 Cc. of Alfalfa Filtrate Representing 23.96 Kilos of Fresh, or 4387 Gm. of Dry, Alfalfa.

		Total N.
	<i>gm.</i>	<i>per cent</i>
Total nitrogen.	55.13	
Ammonia nitrogen.	1.538	2.79
Amide nitrogen.	5.775	10.47
Amino “	14.38	26.08
Solids.	870.4	
Ash.	145.6	

(0.375 gm.). It thus appears that stachydrine occurs in the free condition in alfalfa and not as a constituent of more complex substances.

The yield of choline is also greater than that obtained previously, being 0.050 against 0.025 gm. per gm. of nitrogen in the alfalfa filtrate. Although these variations may be due to differences in the material employed, we are inclined to regard them as the results of greater experience with the methods of base analysis.

EXPERIMENTAL PART.

The "alfalfa filtrate" was prepared from 23.96 kilos of fresh alfalfa gathered May 22, 1924. The plants (leaf and stem) were thoroughly comminuted, pressed out in an hydraulic press, and the clear juice (16.8 liters) was rapidly heated with a current of steam to 81°. The coagulated protein was filtered off and the juice concentrated, under diminished pressure, to 4.6 liters. Alcohol to make a concentration of 50 per cent by volume was then added, the solution filtered, and concentrated to 2500 cc. By this procedure enzyme action was reduced to a minimum and the protein was completely removed together with a large amount of inorganic material. The "alfalfa filtrate" thus obtained gave the results in Table III on analysis.

The fresh alfalfa used contained 222 gm. of nitrogen. Approximately one-quarter of this nitrogen was found in the alfalfa filtrate.

Barium hydroxide was added to the alfalfa filtrate to complete precipitation. The precipitate was removed and an equal volume of alcohol added. After removal of the second precipitate, reagents were removed, the solution was concentrated, and treated with Neuberg's reagent.

The filtrate from the Neuberg reagent precipitate was acidified to Congo red with hydrochloric acid, concentrated, and sodium chloride removed by repeated evaporation with strong alcohol *in vacuo*.

The solution contained 7.023 gm. of nitrogen, 294.8 gm. of organic solids, and 17 gm. of ash.

The bases were precipitated with phosphotungstic acid in the usual way, 320 gm. of reagent being required, and the precipitate was thoroughly decomposed with barium hydroxide. The solution of the bases contained 4.674 gm. of nitrogen.

Isolation of Stachydrine.

Hydrochloric acid equivalent to the nitrogen was added and the solution concentrated *in vacuo*. By successive crystallization from water and alcohol, well crystallized hydrochloride equivalent to 17.197 gm. of free stachydrine was obtained. The mother liquor was a sirup which yielded no further crystalline material on long standing. Since a large amount of stachydrine was subsequently obtained from this sirup, it seems quite possi-

ble that a part of the stachydrine had been esterified by the repeated evaporations with alcohol (11) in acid solution.

The sirupy mother liquor was dissolved in hot water and treated with mercuric chloride until no further precipitate of the quaternary bases could be obtained on concentration and long standing, about 300 gm. of mercuric chloride being required. The filtrate from the mercuric chloride precipitate contained only 0.68 gm. of nitrogen and is discussed below.

The precipitate was decomposed with H_2S , the solution concentrated to a sirup, alcohol added, and several crops of stachydrine hydrochloride were removed, equivalent to 2.706 gm. of the free base. The various crops of stachydrine hydrochloride were combined and recrystallized from water. Melting point, 233–234°C.; nitrogen, 7.64 per cent; theory, 7.8 per cent.

The sirupy mother liquor was dissolved in water, neutralized to litmus with $NaOH$, and precipitated according to Staněk's directions (1) in the presence of 5 per cent of sodium bicarbonate, with potassium periodide reagent. The precipitate separated as heavy green oil which refused to crystallize even when treated with iodine. The solution was allowed to stand overnight and the oil then separated. The aqueous part is discussed below.

TABLE IV.
Composition of the Chloroplatinates from the Choline Fraction.

Crop.	Weight.	Pt	Choline.	Choline chloro- platinat.	Stachydrine chloro- platinat.
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>
1	1.355	28.51	13.2	0.1788	1.176
2	1.223	28.58	15.2	0.1856	1.036
3	0.925	28.30	7.3	0.0676	0.858
4	2.417	28.95	25.5	0.6177	1.800
5	0.744	27.59			
Total.....				1.0497	4.870

The oil was decomposed with copper powder and $CuCl_2$ in the usual way. It contained 1.807 gm. of nitrogen, by far the greater part of the nitrogen in the mother liquor. Subsequent work was carried out on aliquot parts. Since this solution should contain choline it is referred to as the "choline fraction."

Chloroplatinic acid and alcohol were added to an aliquot in small increments and ten successive crops of well crystallized material removed. The first crop contained 28.47 per cent of platinum and the last, 27.74 per cent. This indicated that the greater part of the material was stachydrine chloroplatinate (theory 28.04 per cent). These crops were therefore combined and recrystallized, first from water and then successively from increasing concentrations of alcohol. The composition of the crystals so obtained is given in Table IV.

In addition to this material a portion of the last crop of the first crystal-

lization used for analysis, weighing 0.2581 gm., contained 27.74 per cent of platinum. Assuming that those crops containing platinum in excess of 28.04 per cent were mixtures of stachydrine and choline, the composition of the first four crops has been calculated. The stachydrine chloroplatinate so estimated, *i.e.* 4.87 gm., contains 0.1958 gm. of nitrogen. The aliquot of the choline fraction used for this crystallization contained 0.3346 gm. of nitrogen. Consequently at least 58.5 per cent of the nitrogen of the choline fraction belongs to stachydrine. The somewhat low platinum content of Crop 5 and the other small crop referred to above may indicate the presence either of a little stachydrine ester or of a third base, but it is probable that these samples were essentially pure stachydrine chloroplatinate. Granting this, the yield of stachydrine chloroplatinate was 5.862 gm., containing 0.2356 gm. of nitrogen; hence 70 per cent seems a conservative estimate of the proportion of nitrogen attributable to stachydrine. This corresponds to the presence of 12.92 gm. of free stachydrine in this fraction.

Further evidence that a large part of the base in the choline fraction is stachydrine was obtained by fractional crystallization of the picrates from another aliquot of this solution. Although it appears to be nearly impossible to separate stachydrine from choline by this means, the first crop on recrystallization accounted for nearly 40 per cent of the stachydrine as calculated above, and contained 15.06 per cent of nitrogen (theory for stachydrine picrate 15.05 per cent). The melting point was, however, low. A small additional crop was obtained which melted sharply at 196° and contained 15.0 per cent of nitrogen. This establishes the identity of the material as stachydrine.

Isolation of Choline.

Another equal aliquot of the choline fraction was treated with an excess of sodium bicarbonate and precipitated by Staněk's reagent. The precipitate hardened when iodine was added. This behavior indicates that the precipitate contained but little stachydrine. On decomposition in the usual way and crystallization of the chloroplatinates, 1.3102 gm. were obtained, containing 31.76 per cent of platinum. Theory for choline chloroplatinate, 31.6 per cent. The choline chloroplatinate contained 0.0596 gm. of nitrogen, consequently at least 17.8 per cent of the nitrogen of the choline fraction belongs to choline. This corresponds to the presence of 2.767 gm. of free choline in the whole betaine fraction.

Stachydrine Fraction.

The aqueous solution separated from the oily precipitate obtained with Staněk's reagent was concentrated, acidified with dilute sulfuric acid, and again precipitated with Staněk's reagent. The precipitate, which solidified on standing overnight, was removed and decomposed. It contained only 0.242 gm. of nitrogen. An aliquot of this solution, which contained 0.0896 gm. of nitrogen, was freed from chloride with silver sulfate, reagents were removed, concentrated, dissolved in alcohol, and an equivalent of

picric acid was added. A crop of beautifully crystallized stachydrine picrate, weighing 1.377 gm., was removed. Once recrystallized, this melted at 195–196°C. and contained 14.9 per cent of nitrogen (theory, 15.05 per cent). No further stachydrine picrate could be obtained from the mother liquor. The yield represents 0.0581 gm. of stachydrine nitrogen or 64.7 per cent of the nitrogen in the aliquot used. This corresponds to 1.60 gm. of free stachydrine in the whole fraction.

Picric acid was removed from the mother liquor and chloroplatinic acid added. A crop, weighing 0.1823 gm., of well formed crystals, containing 29.9 per cent of platinum, was obtained. Theory for betaine chloroplatinate, 30.3 per cent. Unfortunately the amount of material available did not permit a more certain identification of this substance. The amount isolated contained 0.00954 gm. of nitrogen or 11.3 per cent of the nitrogen of the stachydrine fraction. This corresponds to a yield of 0.228 gm. of free betaine. Any betaine present in alfalfa should accumulate in this fraction. On account of the insolubility of betaine hydrochloride in alcohol this yield is doubtless minimal, since losses must have occurred when removing sodium chloride from the solution earlier in the analysis.

Filtrate from the Mercuric Chloride Precipitate.

This solution was freed from mercury with H_2S and concentrated *in vacuo* to a sirup which stood in a desiccator for a long time without crystallizing. It was dissolved in water and extracted twice with butyl alcohol to remove the coloring matter which had separated. The extracts were washed with dilute acid and found to contain 0.085 gm. of nitrogen while the aqueous solution contained 0.597 gm. This solution was made alkaline and precipitated with Staněk's reagent. The greater part of the solution was lost by an accident at this point but much of the precipitate was saved. This was worked up in the usual way and 1.361 gm. of a chloroplatinate separated, crystallizing in large orange-red octahedra and containing 37.18 per cent of platinum. Theory for trimethylamine chloroplatinate, 37.01 per cent. A qualitative test demonstrated the volatile nature of the base and the odor of trimethylamine. The yield corresponds to 0.305 gm. of the free base in the alfalfa filtrate, but probably represents only a part of the total amount of trimethylamine. The presence of this base had not been suspected and no steps were taken to prevent losses by volatilization from alkaline solutions during the early steps of the fractionation. Doubtless an appreciable part of the "ammonia nitrogen" of the alfalfa filtrate is to be attributed to trimethylamine. It is by no means certain that the trimethylamine was not produced secondarily from choline in the alkaline solutions employed in the fractionation. This point will be investigated later.

SUMMARY.

A fraction containing the quaternary bases has been obtained from the juice of the alfalfa plant by a method reported in a

previous paper (4). This fraction contained 8.48 per cent of the nitrogen of the protein-free juice of the plant (the "alfalfa filtrate") and nearly 81 per cent of this nitrogen has been accounted for as stachydrine, choline, trimethylamine, and betaine. The yields are given in Table V.

The alfalfa filtrate was not subjected to hydrolysis; these bases, therefore, probably occur, to at least the extent given, free, or as salts, in the juice expressed from the plant and not as

TABLE V.

	Fresh plant.	Dry plant.	N as N of juice.	Substance per gm. N in juice.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>gm.</i>
Stachydrine.....	0.144	0.785	6.09	0.624
Choline.....	0.0115	0.063	0.58	0.050
Trimethylamine.....	0.0013	0.0069	0.13	0.0055
Betaine.....	0.00095	0.0052	0.049	0.0041

components of more complex substances. From the chemical point of view, at least, they are very important constituents of this plant.

Staněk's periodide reagent was found to precipitate considerable stachydrine together with choline at alkaline reaction. Schulze and Trier (7) likewise report this behavior.

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SOME NITROGENOUS CONSTITUENTS OF THE JUICE OF THE ALFALFA PLANT.

V. THE BASIC LEAD ACETATE PRECIPITATE.*

By HUBERT BRADFORD VICKERY AND CARL G. VINSON.

(From the Laboratory of the Connecticut Agricultural Experiment Station,
New Haven.)

(Received for publication, June 10, 1925.)

Basic lead acetate has been employed as a reagent for the clarification of plant extracts for many years. As early as 1826 Garot (1) found that while normal lead acetate removed most of the color from a hot water extract of coffee beans, basic lead acetate was necessary to decolorize the extracts from roasted beans. Schulze almost invariably used basic lead acetate before attempting to isolate the constituents of plant extracts, and the reagent is still in common use (2, 3).

We have failed, however, to find any serious attempt to determine the general nature of the nitrogenous substances precipitated from plant extracts by basic lead acetate. Aside from a foot-note reference by Schulze and Bosshard¹ to the presence of "xanthine bodies" in basic lead acetate precipitates obtained from leaf extracts, very little appears to be known of the nature of the nitrogenous substances in such precipitates.

In this paper are described the results of a preliminary examination of the nitrogenous substances precipitated by basic lead acetate from the concentrated, protein-free juice of the alfalfa

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The preliminary analysis of the hydrolyzed material precipitated by basic lead acetate from alfalfa filtrate was carried out by Mr. Vinson. As he has left this laboratory it was thought advisable to publish the results in their present incomplete state. We hope to return to this problem at a later date.

¹ Schulze and Bosshard (4), p. 440.

plant, referred to as "alfalfa filtrate" in previous papers from this laboratory (5).

The proportion of the total nitrogen precipitated by basic lead acetate from alfalfa filtrate is variable. We have obtained a precipitation of 29.1 per cent of the total nitrogen by adding a large excess of reagent to a concentrated solution of the alfalfa filtrate, while in an experiment on another preparation only 16.0 per cent was precipitated.² Even these figures may not represent the true limits since some nitrogen is held in the lead sulfide obtained by the decomposition of the lead precipitate. Similarly, the proportion of solids precipitated varies, 41.7 and 17.3 per cent being precipitated in the two experiments mentioned above.

TABLE I.

Yields of Substances Isolated from the Hydrolyzed Material Precipitated from Alfalfa Filtrate by Basic Lead Acetate and Representing 44 Kilos of the Fresh Plant.

	Substance.	N
	gm.	gm.
Adenine.....	0.168	0.087
Arginine.....	0.053	0.017
Lysine.....	1.75	0.336
Aspartic acid.....	7.091	0.729
Tyrosine.....	0.368	0.028
Stachydrine.....	1.409	0.137
Total.....	10.839	1.334

Since it appeared inadvisable at this stage to attempt the isolation of the complex substances contained in the lead precipitate, the solution, obtained by decomposing it with hydrogen sulfide, was hydrolyzed with sulfuric acid. The hydrolyzed solution was then fractionated in the manner described in previous papers of this series (5) and the substances listed in Table I were isolated.

These yields have hardly more than qualitative importance since in this preliminary work we necessarily could not deal adequately with the quantitative aspect of the analysis.

The presence of nitrogenous bases in the precipitate obtained

² Unpublished data of Dr. A. J. Wakeman.

with basic lead acetate from alfalfa filtrate, indicates that this reagent must be used with more caution than has been the practice in the past. It seems likely that complex compounds were precipitated which yielded these bases on hydrolysis.

Aspartic acid and tyrosine form insoluble lead salts and their presence in the precipitate is to be expected. Leucine, valine, and phenylalanine, which also form sparingly soluble lead salts, were probably present in the monoamino acid fraction since the greater part of the nitrogen of this fraction was amino nitrogen. A mixture of monoamino acids was obtained, but the quantity was too small to warrant an attempt to separate them.

EXPERIMENTAL.

"Alfalfa filtrate" was prepared from 44 kilos of fresh alfalfa in the usual way and the concentrated solution (5 liters), which contained 80.8 gm. of nitrogen and 1251 gm. of solids, was treated with an excess of basic lead acetate. The precipitate was sucked out dry, whipped up with cold water, and filtered as before. It was then thoroughly decomposed with hydrogen sulfide and the lead sulfide removed. The solution contained 23.5 gm. of nitrogen and 521 gm. of organic solids. After adding 20.7 per cent of sulfuric acid, the solution was boiled for 18 hours and the black humin filtered off. This, when dried, weighed 102 gm. and contained 2.7 gm. of nitrogen. The solution contained 18.76 gm. of nitrogen and 295 gm. of solids after removing sulfuric acid. The thoroughly washed barium sulfate contained 2.3 gm. of nitrogen and must have contained over 120 gm. of organic solids.

The hydrolyzed solution was then precipitated with Neuberg's reagent (6), the precipitate decomposed with hydrogen sulfide, and the solution treated with phosphotungstic acid in the usual way. The filtrate from the Neuberg reagent precipitate was acidified with acetic acid, mercury removed as sulfide, an excess of hydrochloric acid added, and sodium removed as chloride with alcohol. The solution was then concentrated to a sirup, dissolved in water, and likewise treated with phosphotungstic acid.

The solution of the substances precipitated both by Neuberg's reagent and phosphotungstic acid (basic amino acids and purines) contained 2.12 gm. of nitrogen or 11.3 per cent of the nitrogen of the hydrolyzed material precipitated by basic lead acetate, and 11.4 gm. of solids. It was further fractionated with silver sulfate and silver sulfate together with barium hydroxide in the manner described in a previous paper (7).

From the silver sulfate precipitate 0.454 gm. of adenine picrate was obtained which, on recrystallization, melted with decomposition at 295°C.

The silver sulfate and baryta precipitate was worked up for arginine. About one-half of this solution was lost through accident, but from the remaining part, 0.138 gm. of arginine picrolonate, melting at 240°, was obtained.

The filtrate from the silver baryta precipitate was precipitated with mercuric chloride and baryta. The precipitate was worked up for lysine, and 4.493 gm. of the picrate, melting in the characteristic manner at 258° , were obtained.

The solution of the substances precipitated by Neuberg's reagent but not by phosphotungstic acid (monoamino acids), contained 4.5 gm. of nitrogen or 24 per cent of the nitrogen of the hydrolyzed material and 39 gm. of organic solids. The solution was precipitated with calcium hydroxide and alcohol according to Foreman's procedure (8) and the precipitate worked up for aspartic acid. Anhydrous copper aspartate weighing 10.13 gm. was obtained. This contained 7.07 per cent of nitrogen; calculated, 7.20 per cent. The filtrate from the copper aspartate contained over a gm. of nitrogen, most of which was amino nitrogen. Other amino acids were certainly present, but nothing definite could be obtained from it.

The filtrate from the dibasic amino acids, precipitated by Foreman's procedure, contained 2.06 gm. of nitrogen of which over 70 per cent was amino nitrogen. Tyrosine, weighing 0.368 gm., was obtained by direct crystallization; nitrogen, 7.67 per cent; calculated, 7.74 per cent. The mother liquor yielded mixtures from which nothing definite could be separated.

The substances precipitated by phosphotungstic acid from the filtrate from the Neuberg reagent precipitate (quaternary bases), contained 1.69 gm. of nitrogen or 9 per cent of the nitrogen of the hydrolyzed material. The solution was acidified with hydrochloric acid, treated with a hot concentrated aqueous solution of mercuric chloride in large excess, and the precipitate was decomposed with hydrogen sulfide. It contained 0.6 gm. of nitrogen. On concentration 1.76 gm. of stachydrine hydrochloride were obtained, melting at 230° ; nitrogen, 7.69 per cent; calculated, 7.80 per cent. The mother liquors were not further investigated but obviously contained considerable stachydrine.

The bases in this fraction not precipitated by mercuric chloride were not further investigated.

It is interesting to note that over 20 per cent of the nitrogen in the hydrolyzed solution of the substances precipitated by basic lead acetate from alfalfa filtrate belonged to substances giving precipitates with phosphotungstic acid and therefore presumably of basic nature. That so large a part of the nitrogen of these precipitates should be basic nitrogen has not been appreciated in the past and indicates the necessity for further research in this field.

SUMMARY.

The nature of the nitrogenous substances precipitated by basic lead acetate from plant extracts has received very little attention

in the past in spite of the fact that this reagent has been in common use for over a century. It has been employed for the most part to remove objectionable coloring matter from such solutions without regard to the possibility that important nitrogenous constituents might likewise be precipitated.

The precipitate obtained by adding an excess of basic lead acetate to the concentrated protein-free juice of the alfalfa plant was decomposed by hydrogen sulfide and the solution hydrolyzed with sulfuric acid. Appreciable amounts of adenine, arginine, lysine, stachydrine, aspartic acid, and tyrosine have been isolated from this material. It seems possible, therefore, that the bases in this list may have formed components of more complex substances which were precipitated by the reagent. In view of this it is suggested that basic lead acetate should be used as a clarifying reagent for plant extracts with some caution.

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STUDIES ON EXPERIMENTAL RICKETS.

XXVI. A DIET COMPOSED PRINCIPALLY OF PURIFIED FOOD-STUFFS FOR USE WITH THE "LINE TEST" FOR VITAMIN D STUDIES.

BY E. V. MCCOLLUM, NINA SIMMONDS, AND J. ERNESTINE
BECKER

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

AND P. G. SHIPLEY.

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

(Received for publication, June 2, 1925.)

In former papers we discussed the production of rickets by diets low in phosphorus and in vitamin A (1), and a delicate biological test for calcium-depositing substances (2). In preparing animals for this test we employed a diet which we designated Diet 3143, which had the following composition: maize (yellow) 33, wheat 33, gelatin 15, wheat gluten 15, sodium chloride 1.0, and calcium carbonate 3.0 per cent, respectively.

This diet has been employed in several laboratories to produce exaggerated rickets. In later experiments we have met with occasional annoying complications in using this diet. The bones of entire groups of little rats showed sufficient calcium deposits to make it impossible to use them as subjects for the "line test," and this difficulty was traced to the chance employment of *hard* wheat in making up the food formula. Investigation showed that *hard* wheats as a rule had a slightly higher phosphorus content than *soft* wheats and that this small excess of phosphorus was sufficient to modify the composition of the diet when *hard* wheat was used so as to make it of uncertain value. We have not had any difficulty in producing a sufficiently severe grade of rickets in rats to allow them to be used for the line test when soft wheat was used in the formula. Since it is pos-

98 Studies on Experimental Rickets. XXVI

sible that in certain regions of the world soft wheat would not be available we have tried to evolve a diet suitable for making this test which would preclude any element of uncertainty, and which could be used anywhere.

Several diets composed in great measure of purified food-stuffs have been found to produce rickets which would be suitable for the above purpose, but it was deemed of importance to secure one which would be inexpensive and the components of which should be of very constant composition. The following formulas fulfill these conditions.

<i>Diet 4025.</i>		<i>Diet 4026.</i>	
	<i>gm.</i>		<i>gm.</i>
Wheat germ.....	5.00	Wheat germ.....	5.0
Salt mixture 37.....	5.15	Salt mixture 38.....	4.3
Calcium carbonate.....	1.50	Calcium carbonate.....	1.5
Gelatin.....	10.00	Casein*.....	20.0
Egg albumin.....	10.00	Gelatin.....	5.0
Wheat gluten.....	12.00	Wheat gluten.....	5.0
Agar-agar.....	2.00	Agar-agar.....	2.0
Dextrin.....	49.35	Dextrin.....	52.2
Butter fat.....	5.00	Butter fat.....	5.0

* The casein was purified according to the method used in this laboratory and described elsewhere (3).

Diet 4033.

This was the same as Diet 4025 but with the wheat germ replaced by the same amount of yeast (Northwestern Yeast Company Product).¹

Diet 4034.

This diet was the same as Diet 4026 but with the wheat germ replaced by the same amount of yeast (Northwestern Yeast Company product).

Composition of Salt Mixtures.

<i>Salt Mixture 37.</i>		<i>Salt Mixture 38.</i>	
CaCO ₃	1.50	Identical with Salt Mixture	
KCl.....	1.00	37 except that no KH ₂ PO ₄ was	
NaCl.....	1.00	added. The casein in the diet	
NaHCO ₃	0.40	furnished an appropriate amount	
MgO.....	0.20	of phosphorus.	
FeSO ₄ + 7H ₂ O.....	0.20		
KH ₂ PO ₄	0.85		

¹ The authors appreciate the courtesy of Dr. M. H. Givens, of the Northwestern Yeast Company, in supplying the yeast used in these experiments.

In making up the diets the salt mixtures were included in the diets in the amounts indicated. In order to induce rickets of a grade which could be used for the line test procedure an additional 1.5 per cent of calcium carbonate is added to the food mixture as is shown in the descriptions of Diets 4025 and 4026.

If it should be more convenient to use yeast instead of wheat germ as a source of vitamin B, Diet 4034 is the better one of the two diets to use, although the majority of the animals on Diet 4033 would be suitable for line test animals. We have not detected any difference between the effects of Diets 4025 and 4026. The choice of these diets will be determined by convenience in securing materials.

Diets 4025, 4026, and 4034 produce an exaggerated form of rickets. Calcification of the cartilage was entirely wanting in these animals. Young rats weighing 40 to 45 gm. develop severe rickets and are ready to use after being confined to these diets for about 25 days. Those on Diet 4033, however, are usually not ready until they have been on the diet 35 to 40 days. The delay is apparently the result of the inclusion in the diet of the small amount of phosphorus which the yeast contains over that which is added with a corresponding weight of wheat germ.

The rachitic metaphysis is very wide and overproduction of osteoid so great that the bones can be cut as easily as the soft tissues of a normal animal. The type of rickets induced by these diets is what we have termed low phosphorus-high calcium rickets. Thus far we have not succeeded in preparing a diet for the production of low calcium-high phosphorus rickets which induces sufficient growth to make them as satisfactory subjects for this test as the animals prepared by the diets herein described.

Young rats develop ophthalmia after being fed Diets 4033 and 4034 for about 40 to 45 days. If they are used for the line test after being on the diet 25 to 35 days this is immaterial. This ophthalmia is apparently caused by the peculiar effect of feeding too much of the salt mixtures Nos. 37 and 38, respectively. As we have pointed out elsewhere (4) the appearance of the ophthalmia may be due to the dual nature of vitamin B. Provisionally, we must account for its appearance when 5 per cent of yeast is the sole source of this substance, and its failure to develop when 5 per cent of wheat germ serves this purpose, on the assumption that the germ is richer than the yeast in vitamin B.

100 Studies on Experimental Rickets. XXVI

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A COMPARISON OF THE CONCENTRATIONS OF INORGANIC SUBSTANCES IN SERUM AND SPINAL FLUID.*

BY BENGT HAMILTON.

(*From the Medical Clinic of the Children's Hospital and the Department of Pediatrics, Harvard Medical School, Boston.*)†

(Received for publication, June 5, 1925.)

The following investigation was undertaken in the hope that a comparison between blood and spinal fluid in epilepsy might bring to light pathologic changes which studies of the blood alone or the spinal fluid alone have failed to reveal. Measurements were made of total fixed base, calcium, chloride, bicarbonate, and inorganic phosphorus in samples of blood and spinal fluid taken as nearly simultaneously as possible. Early in this study, however, it was appreciated that but little normal data are available with which to compare such measurements. In the work of Pincus and Kramer (1) a complete acid-base balance in the two fluids is given, but some of their findings make it, perhaps, permissible to doubt the accuracy of the sodium measurements.¹ The study was therefore extended to other groups of cases.

Methods.

Determinations in the blood were made on serum, as oxalated or citrated blood could not be used for the determination of calcium and total fixed base. The samples of blood and spinal fluid were, as far as possible, collected simultaneously; they were delivered through a small bore glass tube and under oil into 15 cc. centrifuge

* I wish to acknowledge my very great obligation to Dr. W. G. Lennox for providing me from the Neurological Outpatient Department of the Massachusetts General Hospital with much of the material used in this study.

† The expense of this study was partially defrayed by the New York Committee on Epilepsy.

¹ The average for total anions in spinal fluid was 9 millimols higher than the average for total cations.

tubes, which were filled to the top and stoppered with one-hole rubber stoppers, permitting the expulsion of superfluous oil; the hole was then closed with a glass plug. The *chlorides* were determined by the method of Fiske and Lin (2) on 1 cc. of serum. The error of these determinations is about 1 per cent. The

TABLE I.

Epilepsy.

Values in cc. of 0.1 N in 100 cc. of serum and spinal fluid.*

Case.	Cl		HCO ₃		PO ₄		Total base.		Ca	
	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.
G-i.†.....	103	125	24	25	3.5	0.95	153	157		
Th-l.....	102	127	29	25	2.4	2.3	161	170	5.6	2.6
H-on.....	100	122	25	24	3.0	1.2	158	158	5.3	2.5
M-ck.....	95	119	30	26	2.1	1.0		163	5.0	2.7
B-er.....		122		24		1.0				2.6
N-ia.....	99	123	30	23	1.5	1.0	157	161	5.1	2.8
M-y.....		121	28	25	2.3	0.9	161	169		2.5
D-y.....		117	26	26	2.0	0.8	158	160	5.1	2.6
M-us.....	98	125	31	24	1.6	0.8	156	163	6.4	3.2
R-ng.†.....	101	121	28	23	3.0	1.8	157	155	5.5	2.9
“†.....	101	122	26	25			157	160		2.7
“†.....	99	119	25	22	1.6	0.9	157	157		
B-nt.....	102	125	27	23	2.0	0.9	159	162	5.7	2.8
L. P.....	99	122	28	23	2.3	1.5	157	164		3.1
R-ss.....	102	125	29	24	1.5	0.6	161	163	4.8	2.9
W-ch.....	102	127	27	23	2.2	1.7	154	158		3.3
Gr-n.†.....	100	124	26	22	2.7	1.2	160	156	4.8	2.2
N-or.†.....	101	124	28	22	2.8	1.7	156	158	6.2	2.9
“†.....		121	27	20	2.3	0.6	155	154	5.0	2.7
Mean.....	100	123	27	24	2.3	1.1	157	160	5.4	2.8

* The valency of the phosphorus in the plasma has been taken as 1.8 (Henderson).

† The cases marked with a dagger (†) are children.

bicarbonate measurements were made according to Van Slyke (3). The measurements were often made on 0.5 cc. of serum; the error is, consequently, about 3 per cent; the figures have, therefore, been given without decimals, but when the ratios were calculated one decimal was used. The *inorganic phosphates* were

estimated by the method of Briggs (4). The *total fixed base* was determined by the Fiske (5) method on 1 cc. of serum, and 5 cc. of filtrate were used for the final determination; the error may be estimated as being about 1 per cent. The *calcium* was precipitated as in the Kramer method but always left standing overnight; it was then centrifuged and washed in the usual manner, but instead of making the final determination by permanganate titration, the precipitate was transferred with four 2 cc. portions of water to a platinum crucible, the water evaporated on the water bath, the precipitate ignited, the CaO dissolved in 1 cc. of 0.015 N HCl and titrated back with 0.01 N NaOH (methyl red as indicator). This method was suggested by Dr. Fiske. The *protein* was determined in the serum by micro Kjeldahl and no correction was made for non-protein nitrogen. The figures give, therefore, only an approximate estimation of the plasma protein. pH was determined in separate samples by a modification of Culen's method devised by Hawkins (6).

The values in the serum of the patients with epilepsy were found to be within normal limits (Table I). Expressed in the terms ordinarily employed, they are, per 100 cc. of serum:

Cl.....	0.556-0.602 gm. (as NaCl)
HCO ₃	55-70 vol. per cent
P.....	2.6-6.0 mg.
Total base.....	153-161 cc. 0.1 N
Ca.....	9.6-12.8 mg.

The values in the spinal fluid, expressed in the same terms, were as follows:

Cl.....	0.684-0.743 gm. (as NaCl)
HCO ₃	42-49 vol. per cent
P.....	1.6-4.0 mg.
Total base.....	154-170 cc. 0.1 N
Ca.....	4.4-6.6 mg.

These figures also agree well with the normal, as far as normal data are available (Mestrezat (7), Steiner and Beck (8), Levinson (9), Collip and Backus (10), Egerer-Seham and Nixon (11), Cohen (12), Pincus and Kramer (1), Leicher (13), and others). Normal figures for total fixed base have not been published except as obtained from the sum of the four bases, determined separately.

Such figures must, however, be considered as more or less inaccurate, because of the unreliability of the sodium method. The above values for total fixed base in the blood agree well with our findings in normal cases² and the values for total base in the spinal fluid are more or less the same as those found in a number of other cases (Table II).

Most of the samples were taken between seizures. Case R-ss., however, (*petit mal*) had an attack during the lumbar puncture. The first of the three sets of samples from R-ng. was taken immediately after an attack of general convulsions. In none of these cases do the values show any material difference from the other values in the table, and the same is true of the ratios between the spinal fluid values and the serum values (see Table V). The conclusion seems, therefore, justified that in these seventeen cases of epilepsy a comparison between the serum and the spinal fluid in respect to inorganic constituents gave an entirely negative result as to pathologic findings. When more information has been obtained as to the normal relationship between serum and spinal fluid, this conclusion may, possibly, have to be revised.

The cases tabulated in Table II need no separate discussion.³ The main purpose of these measurements is to furnish additional data for the study of the relationship between the composition of serum and of spinal fluid.

In Table III have been placed together all those cases from Tables I and II where chloride, bicarbonate, and total fixed base have been determined both in serum and spinal fluid. As, however, the presence of a high blood sugar, a high non-protein nitrogen, or a high concentration of protein in the spinal fluid may, possibly, influence any equilibrium which may exist between the two fluids, the cases where those factors were present have been excluded from the discussion. The values in Table III are, for the

² To be published later.

³ It may be noted that both in the epileptics and in the miscellaneous group the values for the total base in spinal fluid are always larger than the sum of the acids. In the epileptics the average difference is about 12 cc. 0.1 N, and this amount of base must consequently be bound by unmeasured acid factors (presumably sulfuric acid and organic acids). If the protein in the plasma is assumed to bind about 12 cc. of 0.1 N of base then in these cases, the amount of base in the plasma bound by the remaining unmeasured acid factors would be about 15 cc. of 0.1 N.

Protein in gm. per 100 cc., other values in cc. of 0.1 N per 100 cc. of serum and spinal fluid.

Case.	Diagnosis.	Cl		HCO ₃		PO ₄		Total base.		Ca		Serum protein.	Plasma pH.
		Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.	Serum.	Spinal fluid.		
A-us.....	Brain tumor (high blood sugar).	95	123	31	29	1.6	0.7	149	160	6.2	3.1		
H-d.....	Brain tumor.	100	126	25	21	2.0	0.9	158	159	4.7			
L-hy.....	Congenital lues* (no cerebral symptoms).	100	124	24	21	1.8	0.6	149	157	4.1	2.4	8.20	
Dr-n.....	Tubercular meningitis.*	87	105	26	20		2.3	143	153				
G-er.....	Nephritis.*	106	123	20	20	2.7	0.9	159	160				
McG-n.....	Uremia.*	110	142	15	16		1.3	166	174	4.1	2.7	6.81	
L-es.....	Convulsions.*	104	123	16	18		0.7	153	154				
M-io.....	Enuresis.*	104	125	19	21			159	158	5.7	2.4		
F-ne.....	Character change after encephalitis.*	102	126	21	20	3.0	1.6	150	152				
F-er.....	Ascites and anasarca of unknown origin.*	121	131	10	13	2.6	0.9	158	155	5.4	2.8	6.25	
Z-an.....	Convulsions* (birth injury).	107	126	19	19	2.1	0.8	157	155	5.1	2.6	6.46	
LeF-e.....	Infantile tetany.*	119	21	20	20	1.74	0.56	140	149	4.0	2.7	6.25	7.37
" (on NH ₄ Cl).....	" "	112	122	17	19	1.15	0.42	151	156	4.0	2.8	6.84	7.32
Z-i.....	" "		122	21	20	1.38	0.50	154	153	4.2	2.5	5.67	7.35
K-y.....	" "	100	116	20	18	1.23	0.54	154	147	3.1	1.8	5.38	7.35
" (on NH ₄ Cl).....	" "		122	17	14	0.87	0.33	141	150	3.2	1.9	6.17	
G-ia.....	" "	107	124	22	22	3.34	0.96	158	158	3.2	2.1	4.50	7.30
" (on NH ₄ Cl).....	" "	119	125	17	17	0.92	0.28	154	154	4.0	2.5	5.69	7.27

* The cases marked with an asterisk (*) are infants or children.

TABLE III.
Relationship between Inorganic Substances in Serum and Spinal Fluid.
 Values in millimols per 1000 gm. of water.

Case.	Cl		HCO ₃		PO ₄		Cl + HCO ₃		Monovalent base.		Ca		Total electrolytes.	
	Serum water.	Spinal fluid.	Serum water.	Spinal fluid.	Serum water.	Spinal fluid.	Serum water.	Spinal fluid.	Serum water.	Spinal fluid.	Serum water.	Spinal fluid.	Serum water.	Spinal fluid.
G-i.....	111	125	25	25	1.9	0.5	137	150	159	154	3.0	1.3	312	321
Th-I.....	110	127	31	25	1.3	1.3	141	152	167	167	2.9	1.3	304	303
H-on.....	107	122	27	24	1.7	0.7	134	146	165	155	2.8	1.4	305	306
N-ia.....	106	123	32	23	0.8	0.6	139	146	163	158	3.4	1.6	303	311
M-us.....	105	125	33	24	0.9	0.4	138	149	161	160	3.0	1.5	307	309
R-ng.....	109	121	30	23	1.7	1.0	139	144	163	152				
".....	109	122	28	25			137	147	163	157				
".....	106	119	27	22	0.9	0.5	133	141	163	154				
B-nt.....	110	125	29	23	1.1	0.5	139	148	165	159	3.1	1.4	308	309
L-P.....	106	122	30	23	1.3	0.8	137	145	163	161				
R-ss.....	110	125	31	24	0.8	0.3	141	149	167	160	2.6	1.5	312	311
W-ch.....	110	127	29	23	1.2	0.9	139	150	160	155				
Gr-n.....	107	124	28	22	1.5	0.7	135	146	167	154	2.6	1.1	307	302
N-or.....	109	124	30	22	1.6	0.9	139	146	161	155	3.3	1.5	305	304
H-d.....	107	126	27	21	1.1	0.5	134	147	165	156				
L-hy.....	107	124	26	21	1.0	0.3	133	145	157	155	2.2	1.2	293	301
G-er.....	114	123	21	20	1.5	0.5	135	143	166	157				
L-es.....	112	123	17	18			129	141	159	152				
M-io.....	112	125	21	21			133	146	164	156	3.1	1.2		
F-ne.....	110	126	23	20	1.7	0.9	132	146	156	149				

purpose of comparison, expressed in millimols per kilo of water. As, however, the water content of the serum was not determined, it is here assumed that 1 liter of serum contained 930 gm. of water. When the protein of the serum was determined, use has been made of Van Slyke, Wu, and McLean's (14) formula, according to which the gm. of water in 1 liter of serum approximately equals $990 - 0.8 P$, where P is gm. of protein per liter of serum. It has not seemed necessary to make any corresponding calculation for the spinal fluid and 1 liter of spinal fluid is assumed to contain 1 kilo of water.

The first point to be determined is whether a change from the average in the concentration of one substance in the serum is accompanied by a change in the spinal fluid in the same direction. Another point of interest is, whether the composition of the spinal fluid is more or less constant than the composition of the serum. And last, if it can be proved that some relationship exists between the two fluids, it remains to be determined whether the distribution of the different substances between serum and spinal fluid follows some known law or theory of membrane equilibrium. It must, however, be borne in mind that the fluid obtained by lumbar puncture has been formed in the ventricles. Any relationship which may exist would probably be a relationship between the arterial blood in the capillaries of the choroidal plexus and the ventricular fluid in the vicinity of the locus of formation. During the passage of the fluid down into the spinal canal changes may possibly take place in its composition and during the time spent in this passage the composition of the blood may have changed. Further, the determinations are made in venous blood, not in arterial. All these factors might tend to obscure any possible relationship.

At the bottom of Table III are given statistically derived data to facilitate the comparison between the two fluids. The correlation coefficient gives the degree of correlation,⁴ the standard deviation gives the degree of variation. The standard deviation is also given in percentage of the mean (variation coefficient).

⁴ A correlation coefficient of 1 means that the correlation is perfect, a correlation coefficient of 0 means that there is no correlation at all. The standard error of the correlation coefficient has, however, also to be taken into account.

The correlation coefficient for the chlorides is 0.40 ± 0.17 . This low degree of correlation may very possibly be accidental and due to the small range of most of the values for serum chlorides; there were only three cases with decidedly abnormal values, and in two of these cases the high concentration of chloride in the serum was the effect of medication, where rapid changes in the serum chlorides might take place without immediate parallel changes in the spinal fluid. The only conclusion which seems permissible from these figures is that slight variations of the serum chlorides are not always accompanied by parallel changes in the spinal fluid. The concentration of chloride in the spinal fluid is more constant than the concentration of chloride in the serum.

The values for bicarbonate are also more constant in the spinal fluid, but here we find a high degree of correlation between the two fluids. When the correlation is as good as it is in this case it may be of interest to express it by means of an equation. This (the regression equation) would here be as follows:

$$Y = 0.46X + 9.7$$

where Y is the most probable spinal fluid bicarbonate corresponding to a serum bicarbonate of X (the spinal fluid bicarbonate being expressed in millimols per liter of spinal fluid, the serum bicarbonate expressed in millimols per kilo of serum water). This equation expresses the relationship between serum bicarbonate and spinal fluid bicarbonate *in this group of cases*; the degree of accuracy may be seen in Table IV. In many of the cases in the literature where serum and spinal fluid bicarbonate have been determined (Egerer-Seham and Nixon (11), Collip and Backus (10), Pincus and Kramer (1)) the values show the same relationship as in this group of cases.

The sum of bicarbonate and chloride has been entered in Table III for reasons which will be discussed later. The correlation coefficient for these values is 0.52 ± 0.14 .

The inorganic phosphorus is very variable both in serum and spinal fluid, but the degree of correlation between the two fluids in respect to phosphorus content is fairly good. The variation coefficient is slightly higher in the spinal fluid than in the serum.

The values for monovalent base have been calculated from the values for total fixed base and the values for calcium. The magne-

sium content of normal serum is only about 1 mm, the error caused by neglecting the magnesium is, therefore, within the limits of analytical error, unless the magnesium content of the serum is abnormally high. In the spinal fluid the magnesium is normally somewhat higher than in the serum, 0.6 to 2.6 mm, according to Barrio (15); these values are, however, not high enough to cause

TABLE IV.

HCO₃, millimols in 1000 gm.

Case.	Serum water.	Spinal fluid found.	Spinal fluid calculated.
G-i.....	25	25	21
Th-l.....	31	25	24
H-on.....	27	24	22
N-ia.....	32	23	24
M-us.....	33	24	25
R-ng.....	30	23	24
“.....	28	25	22
“.....	27	22	22
B-nt.....	29	23	23
L.P.....	30	23	24
R-ss.....	31	24	24
W-ch.....	29	23	23
Gr-n.....	28	22	22
N-or.....	30	22	24
H-d.....	27	21	22
L-hy.....	26	21	22
G-er.....	21	20	19
L-es.....	17	18	18
M-io.....	21	21	19
F-ne.....	23	20	20
F-er.....	10	13	14
Z-an.....	20	19	19
LeF-e. II.....	18	19	18
K-y. I.....	21	18	19
G-ia. I.....	23	22	20
“ II.....	18	17	18

any material difference in the estimated values for monovalent base. In some of the cases the calcium has not been determined and in these cases it has been assumed that the calcium content of the serum and the spinal fluid was normal. The error introduced in this way must also be very small as the calcium constitutes a very small part of the total base.

The correlation coefficient for the monovalent base in serum and spinal fluid is 0.53 ± 0.14 . The small range of the values is probably also in this case responsible for the low degree of correlation. In the serum the base is more constant than in the spinal fluid, and and it is of interest to note that of all the substances determined in serum and spinal fluid the serum base is the most constant. It is equalled in this respect only by the total concentration of electrolytes in the serum.

The correlation between the calcium in the serum and the calcium in the spinal fluid is rather good. In the recently published work of Cameron and Moorhouse (16) we find the correlation between serum and spinal fluid calcium about the same as in these cases. The calcium is about equally constant in serum and spinal fluid.

The last two columns in the table give the sum of all the inorganic constituents which have been determined. The mean of the serum values is nearly exactly the same as the mean of the spinal fluid values and in the individual cases the differences between serum and spinal fluid is nearly always within the limits for analytical error (about ± 4 millimols).

When the ventricular fluid is formed the protein of the plasma is nearly completely held back, the difference in protein concentration between the two fluids is, therefore, considerable. This being the case it may be of interest to investigate whether the different concentrations in which the electrolytes are present in serum and spinal fluid may be explained on the basis of the Donnan theory of membrane equilibrium. According to this theory, $A_f:A_s = B_s:B_f$, where A_f and A_s are concentrations of any monovalent anion or sum of monovalent anions in fluid and serum, and B_s and B_f concentrations of any monovalent cation or sum of monovalent cations in serum and fluid. For divalent ions the square root of the values should be used in the above equation. As it is reasonable to assume that all the substances here in question, with the possible exception of calcium, are dissociated to about the same degree in blood and spinal fluid, the ratios between the total amounts of these substances should be about the same as the ratios between their free ions.

The calcium and the phosphorus may at once be excluded from the discussion as it may be seen at a glance (Table III) that the

total amounts of these substances present in serum and spinal fluid do not conform to the Donnan theory.

As the spinal fluid must be supposed to be formed from arterial blood, 2 millimols have been subtracted from the bicarbonate in the serum in calculating the ratios (this gives, of course, only very approximately an estimation of the arterial bicarbonate). No corresponding correction has been made for the chlorides, as such a correction would make very little difference in the ratios.

It may be seen from Table V that the ratios for chlorides, the ratios for monovalent acid ($\text{Cl} + \text{HCO}_3$), and the ratios for monovalent base are, on the whole, rather constant. The standard deviation of the two ratios last mentioned is only about half as large as the standard deviation for the chlorides. The ratios for bicarbonate taken alone are widely variable. The differences between the ratios for chloride, for bicarbonate, and for monovalent base are in nearly every case greater than can be accounted for by analytical error, but the ratio for chloride plus bicarbonate is nearly always in good agreement with the ratio for monovalent base.

It is rather difficult to draw any definite conclusions from these figures. Probably the constancy of the ratios for chloride and base is of more significance than the lack of agreement between them, as several approximations and assumptions, all of which influence the size of the ratios, have been introduced in the calculations. It is evident, however, that the ratio for the sum of the monovalent acids is more constant and shows better agreement with the ratios for the sum of the monovalent bases than the ratios for the acids taken separately. It may be of interest to point out in this connection that the ratio for potassium, as given in the literature (Pincus and Kramer, McVicar and Ross), is quite different from the ratios for the sum of the monovalent bases as found in these cases.

It may also be of interest that the ratios for the sum of the monovalent acids and for the sum of the monovalent bases agree very closely with the theoretical ratio as calculated by means of Van Slyke, Wu, and McLean's equation:

$$r_{sf} = \frac{2([\text{A}]_s + [\text{BP}]_s)}{[\text{BP}]_f + \sqrt{[\text{BP}]_f^2 + 4[\text{A}]_s([\text{A}]_s + [\text{BP}]_s)}}$$

where r_{sf} is the ratio between the serum base and fluid base (or

between fluid acid and serum acid), $[BP]$ the base bound by protein ($[BP]_f$ may be here neglected), $[A]$ acid, and $[B]$ base. For

TABLE V.

Case.	$\frac{\text{Cl spinal fluid}}{\text{Cl serum}}$	$\frac{\text{HCO}_3 \text{ spinal fluid}}{\text{HCO}_3 \text{ serum} - 2\text{mm}}$	$\frac{\text{Monovalent acid spinal fluid}}{\text{Monovalent acid serum}}$	$\frac{\text{Monovalent base serum}}{\text{Monovalent basespinal fluid}}$
G-i.....	1.13	1.07	1.09	1.03
Th-l.....	1.15	0.85	1.08	1.00
H-on.....	1.14	0.94	1.09	1.06
N-ia.....	1.16	0.78	1.05	1.03
M-us.....	1.19	0.79	1.08	1.01
R-ng.....	1.11	0.82	1.04	1.07
“.....	1.12	0.97	1.07	1.04
“.....	1.12	0.87	1.06	1.06
B-nt.....	1.14	0.86	1.06	1.04
L.P.....	1.15	0.83	1.06	1.01
R-ss.....	1.14	0.84	1.06	1.04
W-ch.....	1.15	0.84	1.08	1.03
Gr-n.....	1.16	0.86	1.08	1.08
N-or.....	1.14	0.79	1.05	1.04
H-d.....	1.18	0.84	1.10	1.06
L-hy.....	1.16	0.87	1.09	1.01
G-er.....	1.08	1.03	1.06	1.05
L-es.....	1.10	1.16	1.09	1.06
M-io.....	1.12	1.14	1.10	1.05
F-ne.....	1.15	0.96	1.11	1.05
F-er.....	1.01	1.56	1.04	1.07
Z-an.....	1.10	1.09	1.07	1.07
LeF-e. II.....	1.02	1.15	1.02	1.02
K-y. I.....	1.10	0.96	1.05	1.10
G-ia. I.....	1.11	1.05	1.08	1.04
“ II.....	0.99	1.09	0.99	1.05
Mean.....	1.12	0.96	1.07	1.04
Standard deviation.....	0.048	0.160	0.026	0.023

a serum and a spinal fluid of normal composition this ratio would be about 1.05.

As to the ratios for bicarbonate it seems probable that no error of analysis and none of the approximations made in the calculations may account for the great variability of this ratio. This assumption is rather strengthened by the fact that instead of a constant ratio the bicarbonates show a very good correlation of another order ($Y = 0.46 X + 9.7$).

Consequently, although indications are not lacking that an equilibrium of the Donnan type may, at least partly, govern the distribution of electrolytes between serum and spinal fluid, it seems probable that the equilibrium is modified by unknown factors.

CONCLUSIONS.

In a series of seventeen cases of epilepsy normal concentrations of chloride, bicarbonate, inorganic phosphorus, total fixed base, and calcium were found in serum and spinal fluid. The study was extended to other conditions with the purpose of getting additional information as to the relationship between the above substances in serum and spinal fluid.

The correlation between the chlorides in the serum and the chlorides in the spinal fluid was not very marked, probably because of the small range of the values in the cases studied. A close relationship was found between the bicarbonates in the two fluids. The correlation between the values for calcium was fairly good and was somewhat less close between the values for inorganic phosphorus. Monovalent base showed about the same low degree of correlation as the chlorides and probably for the same reason.

As regards Donnan's theory of membrane equilibrium, it was found that of the three ratios:

$$\frac{\text{HCO}_3 \text{ in fluid}}{\text{HCO}_3 \text{ in serum}}, \quad \frac{\text{Cl in fluid}}{\text{Cl in serum}}, \quad \frac{\text{Monovalent base in serum}}{\text{Monovalent base in fluid}}$$

the first was very variable, the two last mentioned rather constant. The ratios were in most cases so different that the lack of agreement between them cannot be accounted for by analytical error. It was found, however, that the ratio

$$\frac{(\text{Cl} + \text{HCO}_3) \text{ in fluid}}{(\text{Cl} + \text{HCO}_3) \text{ in serum}}$$

was rather constant and usually in close agreement with the ratio for monovalent base.

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AN EXTRACT OBTAINED FROM THE EXTERNAL BOVINE PARATHYROID GLANDS CAPABLE OF INDUCING HYPERCALCEMIA IN NORMAL AND THYREO- PARATHYROPRIVIC DOGS.

By. A. M. HJORT, S. C. ROBISON, AND F. H. TENDICK.

(From the Department of Chemical Research, Parke, Davis and Company,
Detroit.)

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Vassale (1) in 1905 claimed to have prepared an active parathyroid extract, but failed to describe his process of extraction. There is no available evidence supporting his contentions. Berkeley and Beebe (2) (1909) recovered a nucleoprotein from the bovine parathyroid glands which completely relieved acute parathyroid tetany in dogs. Their process consisted in shaking freshly ground gland tissue at room temperature for 2 hours with 6 to 8 volumes of physiological salt solution to which had been added 2 drops of 10 per cent sodium hydroxide. The resulting product was filtered and preserved with chloroform. This preparation was claimed to be effective orally but better subcutaneously or intraperitoneally. It was, however, not very stable, being readily destroyed by heat and cold and slowly by digestive ferments.

Hanson (3) in a series of papers (1923-24) described a parathyroid preparation which he called "hydrochloric X." It was made by boiling fresh parathyroid glands with 0.1 N hydrochloric acid. He succeeded in relieving parathyroid tetany in dogs for varying periods of time. The same author (4) showed recently that this extract induced a serum calcium increase in thyreoparathyroprivic dogs. Berman (5) reported the preparation of a crystalline substance by means of acid-alcohol extraction of bovine parathyroid glands which possessed the property of inducing hypercalcemia. His report was very brief, and gave nothing in detail. Collip (6), a short time ago, described an active serum calcium-increasing extract of the parathyroids, obtained by boiling

them with hydrochloric acid. His preparation was active when administered to either normal or thyreoparathyroprivic dogs. In general the extracts made by the three foregoing investigators were similar.

Other attempts have been made to prepare active parathyroid extracts, notable among which were those of Massaglia, and MacCallum and Vogel (7). The preparation of the former was composed of a mixture of calcium salts and parathyroid substance, hence making difficult an estimation of its value. MacCallum and Vogel triturated bovine parathyroid glands with Ringer's solution and glycerol, and injected the resulting extracts intravenously in thyreoparathyroprivic dogs. They succeeded in at least temporarily or partially relieving tetany without modifying the blood calcium. They raised the question whether or not some other factor influenced tetany than just the one controlling the blood calcium.

The present writers, hoping to prepare an active serum calcium-raising extract from the external bovine parathyroid glands which would serve for human application, made numerous preparations, and found that the above mentioned activity lay invariably in the acid extracts. The details of this investigation are recorded in the following pages.

EXPERIMENTAL.

I. Preliminary Remarks.

In the following experiments normal and thyreoparathyroprivic dogs were employed in testing the effect of parenterally administered parathyroid extracts on the serum calcium levels. All dogs were kept on a standard diet, fasted for about 16 hours before commencement of experiments, and in the cases where the observations were conducted over not more than 1 day, the animals received neither food nor water during that time. Both male and female, as well as young and old, dogs served as experimental animals.

The calcium analyses were performed according to the technique of Kramer and Tisdall (8). Over a long series of observations normal variations of serum calcium during all hours of the day lay well within 10 per cent in each dog. When the difference was greater, it could be ascribed to some good cause, such as extremely

warm weather or drinking much water. The variation in serum calcium in different dogs ranged from 10.0 to 13.0 mg. per 100 cc. of serum with the great bulk of values occupying a range of from 11.0 to 12.5 mg. Duplicate analyses were always made, and in most instances the 0.01 N potassium permanganate titrations were within 0.01 cc. of each other. Occasionally, 0.02 cc. differences were encountered, and when greater than the latter, the results were discarded. A difference of 0.01 cc. in titration is equivalent to 0.2 mg. of calcium per 100 cc. of serum. But one modification was made in the above method which was to permit a 1 hour contact between the blood serum and the ammonium oxalate instead of a half hour as directed. We could find no appreciable increase in the values obtained by permitting a contact of more than 1 hour.

II. General Comment.

Extracts were prepared from fresh moist and fresh acetone-desiccated bovine parathyroid glands by a number of methods. In each case the ultimate product represented the extractive of a large quantity of gland tissue. As a rule, the extracts were divided into two fractions by precipitation of proteins with neutralization and addition of acetone or alcohol.

The following groups of extracts were prepared and tested for serum calcium-raising properties by parenteral administration in dogs.

A. Inactive Extracts.

1. Extraction of pulped glands with neutral distilled water.
2. Extraction of pulped glands with 65 per cent alcohol.
3. Extraction of pulped glands with 0.1 per cent acetic acid in 40 per cent alcohol.
4. Extraction of pulped glands with 0.5 per cent sodium hydroxide in 65 per cent alcohol.
5. Extraction of pulped glands with 0.4 per cent sodium hydroxide in 40 per cent alcohol.
6. Extraction of pulped glands with 0.4 per cent sodium hydroxide.
7. Extraction of pulped glands with ether
8. Extraction of pulped glands with acetone.

B. Active Extracts (made at room temperature).

1. Extraction of pulped glands, after ether and acetone treatment, with 0.5 per cent hydrochloric acid in 65 per cent alcohol.

120 Extract from Bovine Parathyroid Glands

2. Extraction of pulped glands with 0.3 per cent hydrochloric acid in 40 per cent alcohol.
3. Extraction of pulped glands with 0.3 per cent hydrochloric acid.

C. *Active Extracts* (made by boiling).

1. Extraction of pulped glands by boiling with 0.1 N hydrochloric acid for 15 minutes to 2 hours.
2. Extraction of acetone-desiccated pulped glands by boiling with 0.2 to 0.5 per cent hydrochloric acid for 15 minutes to 2 hours.
3. Extraction of acetone-desiccated and chloroform-defatted pulped glands by boiling with 0.2 to 1.0 per cent hydrochloric acid for 15 minutes to 2 hours.

Activity is apparently absent from all types of extract save the acid-aqueous and acid-alcohol. Hence, the original preparation described by Hanson and the acid-alcohol extracts of Berman contain the calcium-controlling hormone of the parathyroids. The recent work of Collip substantiates these results. The positive findings of the present writers are recorded below.

III. *Serum Calcium-Increasing Extracts of Fresh Moist Bovine Parathyroid Glands and Their Effects on Thyroparathyroprivic and Normal Dogs.*

A. *Preparation in Acid Solutions at Room Temperature.*

(1). *Alcoholic Hydrochloric Acid Extract*.—112 gm. of fresh frozen parathyroid glands were finely ground and extracted 16 hours at room temperature with 300 cc. of 0.3 per cent hydrochloric acid in 40 per cent alcohol. Precipitation of colloids was induced by neutralization to the isoelectric point and the addition of 1 volume of acetone. The preparation was then filtered, the clear filtrate concentrated *in vacuo*, and later dried on a hot air bath at 50–60°C. Yield 1.8 gm. 0.5 gm. of this extract was dissolved in 8 cc. of physiological salt solution and injected subcutaneously in normal dog No. 43.

(2). *Aqueous Hydrochloric Acid Extract*.—140 gm. of fresh parathyroid glands were finely ground and extracted 16 hours at room temperature with 300 cc. of 0.3 per cent hydrochloric acid. The preparation from this point was treated identically as in (1) above. Yield 3.5 gm. 1 gm. of this product was dissolved in 8 cc. of physiological salt solution, and injected subcutaneously in normal dog No. 53.

(3). *Which Tissue Constituent Is the Source of the Activity?*—60 gm. of fresh parathyroid glands were carefully trimmed of extraneous fat, and the clean pulp finely ground. Yield 23 gm.

(a). The clean finely ground pulp was extracted for 16 hours at room

temperature with 100 cc. of ether to which had been added 3 drops of concentrated hydrochloric acid. The supernatant ether was then decanted, the residue was washed with two small portions of fresh ether, the ether washings were united with the original solvent, and the total was evaporated to dryness on a hot air bath at 50–60°C. Yield 3.0 gm. This extract was emulsified with about 10 cc. of water, and injected subcutaneously in normal dog No. 57.

(b). After the ether extraction, the residue was extracted for 4 hours at room temperature with 100 cc. of acetone. Then the acetone was decanted, the residue washed with two small portions of fresh acetone, the acetone washings were united with the original acetone extract, and the total was evaporated *in vacuo* to remove all of the acetone. Yield 10 cc. of aqueous solution. This was then injected subcutaneously in normal dog No. 58.

(c). After ether and acetone extraction the undissolved gland residue was digested for 16 hours at room temperature with 100 cc. of 0.5 per cent hydrochloric acid in 65 per cent alcohol, neutralized, filtered, and the filtrate concentrated to a volume of 5 cc. *in vacuo*. This product was injected subcutaneously in normal dog No. 59.

The results of the foregoing observations are described graphically in Chart 1.

B. Preparation in Acid Solutions by Boiling.

(1). 30 gm. of fresh parathyroid glands were finely ground, boiled 15 minutes with 500 cc. of 0.1 N hydrochloric acid, cooled, freed from fat by skimming, and filtered. The pale yellow, hazy filtrate was bottled and preserved in the ice chest. 5 cc. of this filtrate were injected intramuscularly in thyreoparathyroprivic dog No. 77.

(2). Identical preparation with B (1) above save for boiling 2 hours instead of 15 minutes. (Hanson's hydrochloric X.) 5 cc. of this filtrate were administered intramuscularly in thyreoparathyroprivic dog No. 80.

The results of these two experiments are recorded in Chart 2.

IV. Serum Calcium-Increasing Extracts of Fresh Acetone-Desiccated Parathyroid Glands and Their Effect on Normal Dogs.

A. Comparison of Hot and Cold Acid Extracts.

(1). *Extraction with Acid at Room Temperature.*—5 gm. of acetone-desiccated fresh pulped glands were extracted for 4 hours at room temperature with 100 cc. of 0.2 per cent hydrochloric acid, filtered, excess protein was removed by adding strong sodium hydroxide to the isoelectric point, filtered again, and the clear filtrate concentrated to 10 cc. *in vacuo*. To the somewhat turbid concentrate, 2 cc. of 0.1 N sodium hydroxide were added to aid solution. All of the product was injected subcutaneously in normal dog

122 Extract from Bovine Parathyroid Glands

No. 69. (1 gm. of acetone-desiccated tissue is equivalent to approximately 7 gm. of fresh moist glands.)

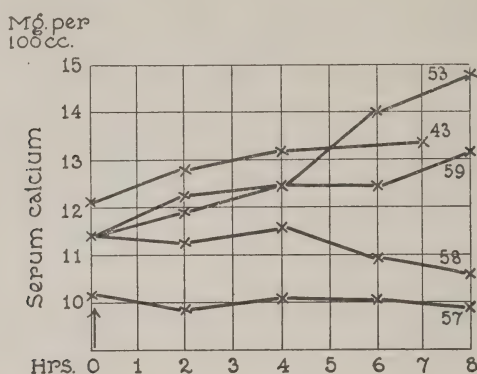


CHART 1. Injections made at time designated by arrow.
Maximum serum calcium increase in Dog 43, 10.7 per cent.
" " " " " " 53, 29.6 " "
" " " " " " 59, 16.0 " "

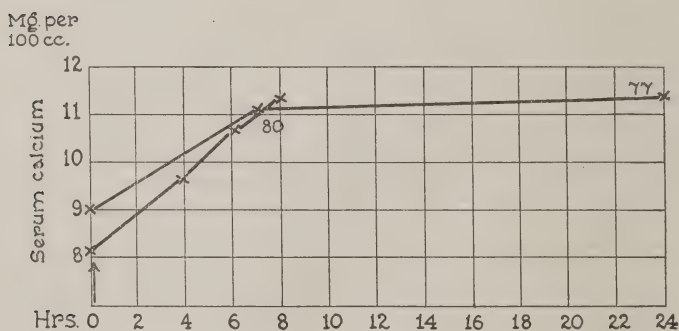


CHART 2. Injections made at time designated by arrow.
Maximum serum calcium increase in Dog 77, 7-24 hrs. 26.7 per cent.
" " " " " " 80, 8 " 40.1 " "

(2). *Extraction with Acid by Boiling.*—5 gm. of acetone-desiccated glands (same lot as used in (1) above) were boiled for 15 minutes with 100 cc. of 0.2 per cent hydrochloric acid, cooled, filtered, and the extract was completed exactly as in A (1) above. Final volume was 14 cc., all of which was administered subcutaneously in normal dog No. 70.

The results of the above two experiments are recorded in Chart 3.

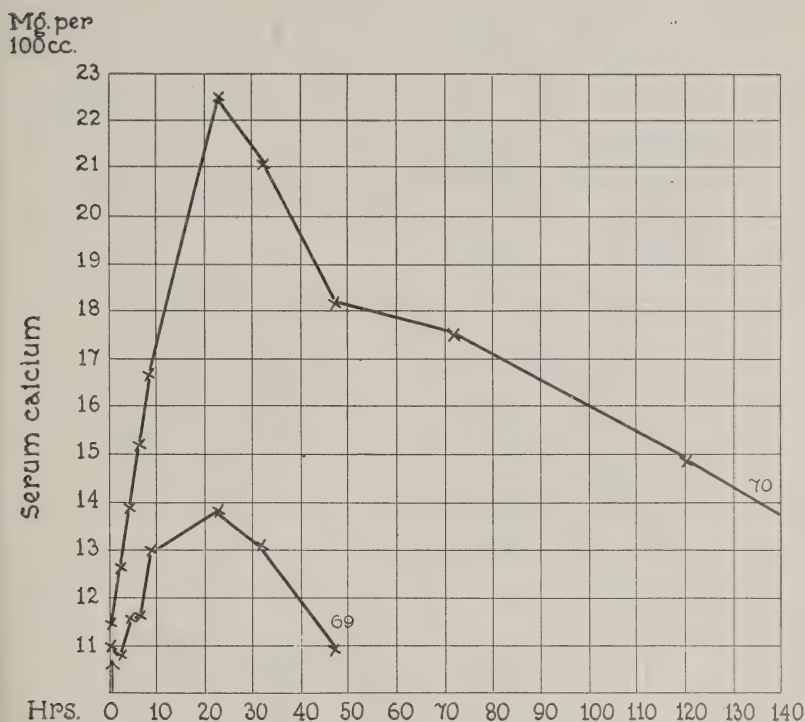


CHART 3. Injections made at time indicated by arrow.

Maximum serum calcium increase in Dog 69, 18-24 hrs. 27.9 per cent.

" " " " " 70, 18-24 " 97 " "

Last serum calcium analysis, 12 mg. at 168th hr.

B. Comparison of Similar Extracts Obtained by Boiling Acetone-Desiccated, and Acetone-Desiccated Chloroform-Defatted Tissues with Dilute Hydrochloric Acid.

(Acetone-desiccated glands lose about 40 per cent of their weight by continuous chloroform extraction.)

(1). 1 gm. of acetone-desiccated glands was boiled with 20 cc. of 0.2 per cent hydrochloric acid for 15 minutes, cooled, and filtered. The excess protein was removed from the filtrate by the addition of strong sodium

124 Extract from Bovine Parathyroid Glands

hydroxide to the isoelectric point and subsequent filtration. The resulting filtrate was evaporated to dryness in a vacuum autoclave, and the desiccated product dissolved in 10 cc. of sterile water. 5 cc. of this solution were injected subcutaneously in normal dog No. 74.

(2). 1 gm. of acetone-desiccated chloroform-defatted glands (same lot of glands as above) was boiled for 15 minutes with 20 cc. of 0.2 per cent hydrochloric acid, cooled, and filtered. The preparation was completed identically as in B(1) above, and 5 cc. of the resulting solution were given subcutaneously in normal dog No. 75.

The results of these observations are recorded in Chart 4.

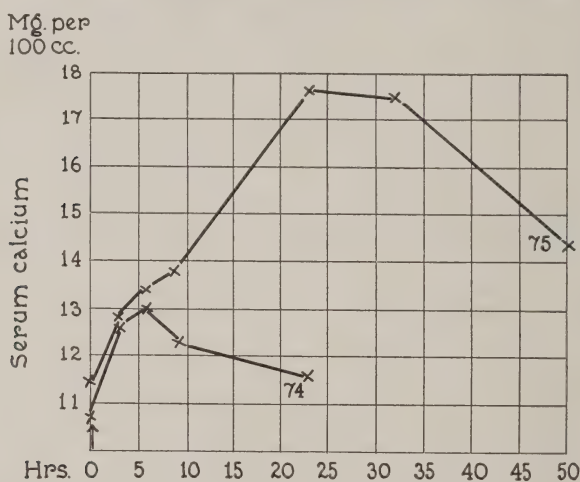


CHART 4. Injections made at time designated by arrow.
Maximum serum calcium increase in Dog 74, 6-9 hrs. 21.5 per cent.
" " " " " " 75, 18 " 54.4 " "

V. Effect of Purification of the Parathyroid Extracts on Their Potency.

10 gm. of finely ground fresh acetone-desiccated and chloroform-defatted parathyroid glands were boiled with 250 cc. of 0.1 N hydrochloric acid for 30 minutes, and then cooled to room temperature. 10 cc. of the filtrate were obtained for testing the potency of the crude product. 2.5 cc. portions of this filtrate were injected intramuscularly in normal dogs Nos. 83 and 84.

To 220 cc. of the unfiltered crude product, strong sodium hydroxide was added to the point of maximum precipitation, and 1175 cc. of 95 per cent alcohol were added to further coagulate proteins. This mixture (approximately 80 per cent alcohol) was permitted to stand 16 hours, and then filtered.

The clear filtrate was concentrated *in vacuo* to remove all of the alcohol. It was then made up to the original volume of 220 cc. with distilled water and preserved with chloretone. 2.5 cc. portions of this preparation were administered intramuscularly to normal dogs Nos. 85 and 86.

These experiments are recorded in detail in Chart 5.

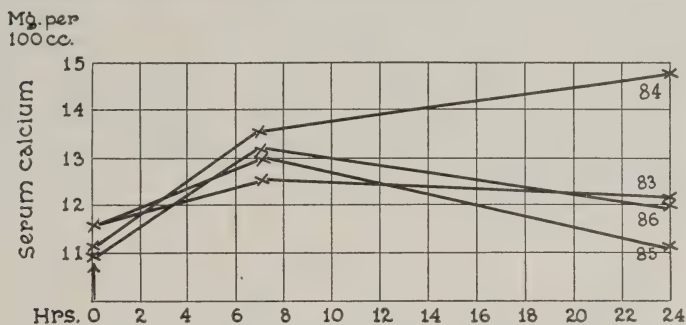


CHART 5. Injections made at time designated by arrow.
 Maximum serum calcium increase in Dog 83, 7 hrs. 8.6 per cent.
 " " " " " " 84, 7-24 " 33.33 " "
 " " " " " " 85, 7 " 12.0 " "
 " " " " " " 86, 7 " 20.0 " "

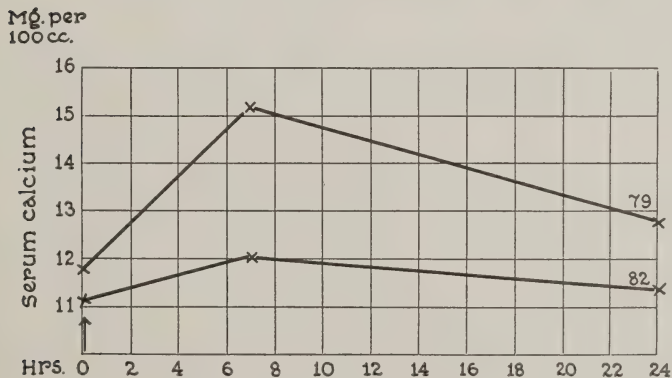


CHART 6. Injections made at time designated by arrow.
 Maximum serum calcium increase in Dog 79, 7 hrs. 28.8 per cent.
 " " " " " " 82, 7 " 7.2 " "

VI. Effect of the Age of the Extract on its Potency.

A preparation was made 16 months ago according to the method of Hanson wherein 30 gm. of freshly frozen and trimmed

parathyroid glands were boiled for 2 hours with 500 cc. of 0.1 N hydrochloric acid, cooled, freed from fat by skimming, filtered, and preserved in the ice chest. Before employing this material for testing it was neutralized to the point of maximum precipitation with strong sodium hydroxide, and further coagulated by the addition of 1 volume of 95 per cent alcohol. This mixture was then filtered, and the clear filtrate concentrated on the steam bath to one-tenth of its original volume. The resulting slightly turbid amber-colored solution was preserved with chloretone. 5 cc. portions of this product were injected intramuscularly into thyreoparathyroprivic dogs Nos. 79 and 82 with the results tabulated in Chart 6.

DISCUSSION.

In Section III A and Chart 1 are presented evidence indicating that the bovine parathyroid glands contain an acid-extractable hormone capable of inducing hypercalcemia in dogs. It further shows that the active substance is probably obtainable from the lipoid-free constituents of the glands, and is rendered soluble in both alcoholic- and aqueous-acid media although alcohol is in nowise essential.

Boiling the finely ground glands with dilute hydrochloric acid is as good if not a better means of extracting the hormone than digestion at room temperature as is demonstrated by Section III B and Chart 2. The hormone is active in increasing the serum calcium of thyreoparathyroprivic as well as of normal dogs. In fact, we have evidence indicative of greater responsiveness of the former to the action of the hormone. The active substance must be quite stable when it is not affected by boiling with acid.

Acetone-desiccated fresh parathyroid glands are as serviceable as the fresh moist gland for the preparation of extracts as is shown in Section IV A and Chart 3. In this section, the evidence suggests that boiling with acid is preferable to extraction at room temperature. Dog 70, although its serum calcium was increased practically 100 per cent, showed no symptoms attributable to the change. Furthermore, large doses are shown to exert their influence over a relatively long period as is here demonstrated, the serum calcium decreasing slowly over a week's time.

The contents of Section IV B and Chart 4 corroborate the obser-

vations recorded in Chart 1 wherein it is suggested that the lipid constituents of the glands are not the source of the hormone. In these two experiments, it is shown that lipid-free tissue yields a very potent extract by boiling with dilute hydrochloric acid. The difference in response between Dogs 74 and 75 to these two extracts may be due to the fact that the acetone and chloroform, desiccated and defatted, material contains more (about 40 per cent), potentially active tissue per gm. of weight than does the acetone, desiccated and defatted.

In Section V and Chart 1 is evidence indicating that most of the protein bulk may be removed without appreciably affecting the potency of the extract. It is possible that some may be lost by precipitation of the colloids for the hypercalcemia test is not sufficiently accurate to detect small losses. The results also show the variation in response of different dogs to the same dose of the hormone. Unrecorded data show this variation to be quite conspicuous, and that in general, dogs which respond well at one time, do so at other times and that dogs which respond poorly, also do so consistently. Certain observations point to the fact that young dogs are most responsive, yet there is considerable variability among them. There is also a suggestion that response depends to some degree, at least, on readily available calcium, for when relatively small amounts of calcium lactate are given orally at the time of injection of the hormone, there is a rapid increase in the serum calcium.

The parathyroid hormone is very stable even when preserved in 0.1 N hydrochloric acid for a period of over a year as is shown by the data in Section VI and Chart 6. In this case, a preparation, made according to the method of Hanson, was preserved for 16 months in the ice chest, and was then still very active. The difference in response between Dogs 79 and 82 shows that the variation existing in normal dogs is shared by thyreoparathyroprivic dogs. In some unrecorded data, operated dogs have shown variations in response at different times. There is some indication that hypoparathyroid dogs with a moderate degree of hypocalcemia respond most readily to injections of the hormone. Some dogs in tetany have been relieved of their symptoms without showing any serum calcium increase, substantiating MacCallum and Vogel's observations, and other dogs or the same dogs at other

times have shown a very rapid increase in their serum calcium simultaneous with relief from symptoms.

The observations with boiled hydrochloric acid extracts corroborate the results published originally by Hanson and later by Collip. The results with acid-alcohol extracts verify the work of Berman. The relative stability of the hormone to treatment with boiling acid and long preservation in the ice chest serve as contradictory evidence against the claims of Berkeley and Beebe.

CONCLUSIONS.

1. A hormone is obtainable from fresh bovine parathyroid glands by aqueous or alcoholic hydrochloric acid extraction which when given parenterally to dogs possesses the property of relieving tetany and inducing hypercalcemia.

2. Boiling the glands with dilute hydrochloric acid is preferable to extraction at room temperature.

3. The lipid-free portion of the glands is the potentially active tissue.

4. Very little, if any, potency is lost in the removal of proteins by neutralization to the isoelectric point and addition of alcohol to a concentration of 80 per cent.

5. The hormone is relatively stable as judged by the vigorous treatment it withstands in the course of its preparation, and its retention of activity during 16 months preservation in the ice chest.

6. Definite conclusions are in some cases not justified because of the limited accuracy of the hypercalcemia test.

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DIRECT PRECIPITATION OF CALCIUM IN COWS' MILK.

By CARMEN S. ROTHWELL.

(From the Department of Pediatrics, School of Medicine, Yale University, New Haven.)

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The standard methods for determining calcium in milk are those of McCrudden (1) and the Official Agricultural Chemists (2). Both involve ashing. Procedures which do not involve ashing are those of Baier and Neumann (3), Halverson and Bergeim (4), and the nephelometric method of Lyman (5). In none of these is there direct precipitation. The success of Kramer and Tisdall's (6) method for precipitating calcium in blood serum without removal of proteins, suggested the possibility of using similar technique for milk. We have, therefore, devised a method of direct precipitation from whole milk, because such a procedure reduces materially the time and difficulty of the determination.

Procedure for Cows' Milk.

To about 2 cc. of distilled water in a 15 cc. centrifuge tube add 1 cc. of whole fresh milk from a certified pipette, graduated to deliver between marks. The contents are mixed by tapping the bottom of the tube with the forefinger, imparting a circular motion. Add 2 cc. of a saturated solution of ammonium oxalate and mix as before. Allow to stand $\frac{1}{2}$ hour. Mix at intervals. Centrifuge for 5 minutes at 1500 R.P.M. Pour off the supernatant fluid, taking care to remove the plug of cream which rises to the top. The last drop is removed with a clean towel, as recommended by Tisdall (7). Add 2 cc. of 2 per cent ammonium hydroxide and 1 cc. of ether, centrifuge, and repeat washing twice, making three washings in all. Dissolve the precipitate in 2 cc. of normal sulfuric acid, heat to 70°C., and titrate against standardized permanganate.

Direct precipitation was in all cases checked against ashing. Our procedure was to evaporate and ash 10 cc. of milk in platinum in an electric oven, dissolve the ash in 0.1 N HCl, neutralize to pH 4.4 to 5.6, using methyl red as an indicator, as recommended by Shohl (8). The volume was made up to 100 cc.; a 2 cc. aliquot was taken for the determination, precipitated

with 2 cc. of saturated ammonium oxalate, centrifuged, and washed as usual. The precipitate was titrated against permanganate solutions, standardized against Sørensen's sodium oxalate.

RESULTS.

The results show that the calcium of milk can be correctly determined by the procedure, as outlined. As much as 10 cc. of milk may be utilized without affecting the accuracy of the

TABLE I.
Determination of Calcium in Cows' Milk by Ashing and by Direct Precipitation.

Sample No.	Ash.	Direct precipitation.	Remarks.
	mg. per 100 cc.	mg. per 100 cc.	
1	127.0	127.0	Direct precipitation of 10 cc. of milk.
	127.0	132.0	" " " 10 " " "
2	119.5	124.4	" " " 1 " " "
	125.6	124.0	" " " 1 " " "
	125.0	124.0	
		135.5	10 mg. Ca added.
3		135.5	10 " " "
	136.6	136.0	Direct precipitation of 1 cc. of milk.
4*	118.8	118.8	" " " 1 " " "
	118.7	118.8	
	120.0	120.0	
	119.3	118.8	
	116.8	117.5	
	124.0		
5		124.0	" " " 1 " " " (boiled).
		124.0	" " " 1 " " " (unboiled).
Average...	123.1	123.7	

* Figures for separate determinations are given for milk sample No. 4, in order to show variations obtained by the two methods, ashing and direct precipitation.

results. Added calcium is recovered quantitatively. Results, given in Table I, by the direct method agree within an average of ± 0.5 per cent to values obtained by ashing. The maximum

variations of the separate determinations agree within 2 per cent and show no greater variations than separate ashings on the same sample of milk. For fresh milk, boiled or unboiled, direct precipitation gives results as accurate as ashing. Contrary to the findings of Lyman (5) who reported low results for whole milk by direct precipitation, we are able to precipitate calcium quantitatively from whole cows' milk. The only practical difficulty encountered is from the presence of fat, which gives turbid solutions and indefinite end-points. The fat may be removed by washing with a mixture of ether and ammonium hydroxide.

The success with cows' milk suggested that the same procedure could be adapted to human milk. As before, the results were checked by determinations on the ash. Determinations on the ashed sample were closely agreeing duplicates or triplicates. Direct determinations also were done in duplicate or triplicate. Results obtained with sour milk were much lower than with fresh milk. The values obtained by the method as outlined for cows' milk were on an average 29.4 per cent low.

Because the results obtained by direct precipitation were unsatisfactory, the following procedures were tried.

1. Deproteinization, according to Folin's method, with sodium tungstate and sulfuric or hydrochloric acid was attempted. This procedure gave closely agreeing results, but they were 12.6 per cent low compared to ashing. Trichloroacetic acid deproteinization, as recommended by Lyman (5), was tried, but we had difficulty in removing all the proteins by this reagent, particularly with old or sour breast milks. Such filtrates gave low results.

2. 5 cc. of saturated ammonium oxalate were added, and the tubes heated to 60°C. in order to favor the precipitation of calcium, but correct results were not obtained. Washing first with saturated ammonium oxalate, followed by 2 per cent ammonium hydroxide, was tried, in order to obviate any error arising from solubility of calcium oxalate, but this procedure either gave low values or erroneous high results due to incomplete removal of the ammonium oxalate. The values varied from +43.5 to -31.5 per cent.

3. An effort was made to regulate the acidity, as recommended by McCrudden (1), by precipitating with oxalic acid and buffering with sodium acetate. Extremely low results were obtained, with an average of -61.6 per cent. 1 to 2 drops of 2 per cent ammonium hydroxide were added, but this, also, gave low results, averaging -44.4 per cent. In the further effort to regulate pH, 1 or 2 drops of 0.1 N HCl were added. The pH resulting from a mixture of milk and ammonium oxalate may be so alkaline as to permit some of the calcium to be precipitated as phosphate and so be

lost in a titration as oxalate. These determinations varied from -2.7 to -20.0 per cent, with an average of -9.0 per cent.

Added calcium chloride was not recovered satisfactorily by any of the direct methods tried. We cannot, therefore, recommend any of the procedures for breast milk, for while many of the values are not more than 1 per cent high or 0.2 per cent low, others may show great errors. With our best procedure on breast milk our error was -9.0 per cent, and did not give the excellent results obtained by the direct precipitation of cows' milk.

We are in agreement with Clark and Collip (9) that the conditions underlying the determination of calcium in such materials as blood hold also for milk; namely, that the method of determination is without adequate basis in theory and represents a carefully standardized empirical procedure, in which the errors of loss of calcium oxalate by solution and failure to remove excess oxalate in the supernatant fluid balance one another. Such a procedure has been approximated for breast milk, but attained for cows' milk.

CONCLUSIONS.

1. Calcium may be accurately determined in cows' milk by direct precipitation with ammonium oxalate, without removal of protein.

2. As yet no method for direct precipitation of calcium from human milk has been satisfactory.

3. Protein-free filtrates from human milk show low results, corresponding, in most cases, with those shown by direct precipitation on the same sample.

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TEMPERATURE COEFFICIENTS OF ENZYMIC ACTIVITY AND THE HEAT DESTRUCTION OF PANCREATIC AND MALT AMYLASES.*

By DONALD H. COOK.

(*From the Department of Chemistry, Columbia University, New York.*)

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There are in the literature many references to the action of heat on enzymic processes. In general these are not specific, and where they are they deal, as a rule, with one isolated point such as the temperature of optimum activity or that of destruction. Not much reliance can be placed on the data given in some of the earlier work (1, 2), as at that time very little was known as to the conditions for the optimum activity of enzymes. The effect of heat on enzymic activity through a wide range of temperature has been little studied. As enzymes are, in general, unstable substances, the effect of heat upon any reaction catalyzed by them is made up of a twofold effect. Euler (3) points out that a rise in temperature first accelerates the chemical velocity and second, inactivates the enzyme. Thus in plotting curves of the activity of an enzyme with increase in temperature the curve always tends to rise to a maximum. Sørensen (4) says: "Therefore, the curve of the temperature should be considered as if it were two entirely different curves, namely, a curve of the real temperature effect which increases in proportion to the temperature elevation, not only below but probably above the optimum temperature, and on the other side a curve of the destruction of the enzyme." The maximum of this temperature curve varies according to the enzyme employed, the acidity, concentration of the substrate, presence of salts, type of solvent, etc. Much of the confusion arising from apparently conflicting data in the literature is due to the lack of fulfilling the conditions of optimum activity of the enzyme,

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especially in regard to hydrogen ion concentration and activating salts. Much of the work of various investigators cannot be compared quantitatively because of failure to insure the optimum hydrogen ion concentration. At any other hydrogen ion concentration the enzyme is less active and also, according to Lüers and Lorinser (5), less stable. Sherman, Thomas, and Baldwin (6) determined the hydrogen ion concentrations at room temperature which enable pancreatic and malt amylases and taka-diaxase to exert their optimum activities when acting upon soluble starch at 40°C. for 30 minutes under definitely specified conditions.

It has been stated as a general rule that for every 10°C. rise in temperature, the velocity of a chemical reaction increases two- to threefold. Harcourt and Esson (7) found for the liberation of iodine from HI by H_2O_2 that between 0° and 50° the velocity was about doubled for every 10° rise. Hudson and Paine (8), working with invertase, found the coefficient of destruction by acids and alkalies was between 2 and 3 for every 10° for the interval 0–45°. Auld (9) working with amygdalin emulsion showed a temperature coefficient of 2 for every 10° between 15–35°C. Vernon (10), using starch and amylase, found that the velocity of hydrolysis was twice as great at 30° as at 20°. Kendall and Sherman (11) give 2 as the approximate temperature coefficient of pancreatic amylase for an interval of 10° between 20° and 40°.

In the light of this previous work it appeared that it might be of interest to study the effect of temperature not only on the speed of hydrolysis but also on the rate of inactivation of two typical starch-splitting enzymes. Pancreatic and malt amylases were chosen for investigation.

Materials Employed.

The starch used in this work was Merck's "soluble starch according to Lintner." It was washed nine times with distilled water, six times with triply distilled water, and air-dried.

Ordinary distilled water was twice redistilled, first over alkaline permanganate solution, then over very dilute solution of orthophosphoric acid; the distillate was condensed in a block tin condenser. This product was always used for making up starch and stock solutions and for the final rinsing of all glassware.

The primary and secondary phosphates used as buffers were purified according to the unpublished methods of Beans and Kiehl. The sodium chloride used was twice recrystallized from triply distilled water and dried at 120°C.

The two enzyme preparations were commercial products: Parke, Davis and Company pancreatin, laboratory sample No. 9, and a barley malt obtained from the Froedtert Malting Co. The malt extract was prepared by grinding 100 gm. of malt and extracting with 250 cc. of triply distilled water at ice box temperature for 2 hours. The extract was then filtered and kept in the refrigerator in a steamed Nonsol bottle. The total solids and reducing substances present in the extract were determined for each new preparation. This stock solution of malt extract was tested from day to day and did not change in activity during the time of the experiments with it. The pancreatin was prepared fresh for each day's experiment and was made up by weighing out 50 mg. of the dry substance and making up to a definite volume with a cold solution of phosphate of the required concentration. The activities of these preparations were determined according to the method of Sherman, Kendall, and Clark (12). The pancreatin had an average activity of 300 on their scale and the malt extract an activity of 48.

EXPERIMENTAL.

1 per cent starch solutions were used as the substrates in these experiments. They were adjusted to pH 7 for the work with the pancreatin and contained 0.05 molar sodium chloride and 0.0005 molar disodium phosphate. In the experiments with malt amylase the starch solutions contained 0.03 molar dihydrogen phosphate and were adjusted to pH 4.5.

The measurements of pH were made either electrometrically or colorimetrically at room temperature (about 22°C.). Although the above conditions, which were chosen because they had been shown by Sherman and coworkers to give the best results with these enzymes in tests of activity carried out at 40° for experimental periods of 30 minutes, may not represent the optimum conditions for testing the activities of these enzymes at other temperatures and for different periods of time, they give a basis for quantitative comparisons of the influence of temperature when starting with the same standard initial conditions.

The direct tests of activity at the various temperatures were carried out as follows: The starch solutions prepared as described above were thoroughly mixed and poured into 100 cc. measuring cylinders which were then immersed in the thermostat at the temperature at which the digestion was to be carried out. 50 mg. of the enzyme preparation, in the case of pancreatin, were then weighed out and made into a smooth paste with a drop of cold water containing the same concentration of sodium chloride and disodium phosphate as the starch solution. This was then washed into a 100 cc. volumetric flask with the cold water-salt solution, made up to the mark, and kept at 10° or colder until used. In the case of malt extract, 2.5 cc. of the extract were made up to 100 cc. with cold triply distilled water without salts. Varying amounts of these enzyme solutions, depending on the activity, were pipetted with an accurately calibrated 1 cc. pipette into dry 200 cc. Erlenmeyer flasks. The starch mixtures in the cylinders were then poured into the flasks at 15 second intervals and the flasks immersed in the bath for $\frac{1}{2}$ hour. At the expiration of this period 50 cc. of Fehling's solution were added to each flask at 15 second intervals and in the same order in which the starch was poured on the enzyme. The flasks were then immersed in a boiling water bath and held at the boiling point for 15 minutes. The cuprous oxide formed was rapidly filtered through Gooch crucibles, washed with hot water, alcohol, and ether, dried at 100°C., and weighed. Maltose was calculated from Defren's table.

The temperatures chosen were 20°, 30°, 40°, 50°, 60°, and 70°C. As a rule five determinations were made simultaneously, four at a lower temperature and one at a temperature 10° higher. Thus, each new series of experiments overlapped the previous work and was a constant check on the technique and the activity of the enzyme. Blank experiments were made not only with the enzyme preparations, but also with the starch and reagents in each series of experiments.

As the presence of starch appears to exert a protective action upon these enzymes it was also of interest to study the influence of changes in temperature upon the enzymic activities when the solutions were held at the various temperatures in the absence of the substrate before being allowed to act upon it.

For these inactivation experiments the above procedure was modified as follows: 300 cc. of a 2 per cent starch solution containing the molar concentrations of sodium chloride and sodium phosphate given above were prepared and placed in 50 cc. portions in the thermostat. 300 cc. of a water-salt solution containing the same concentrations of these salts were also prepared. As both the water-salt solution and the starch solution contained the same concentration of activating salts they would give the standard 1 per cent substrate upon being combined. The water-

salt solution was divided into 50 cc. portions which were immersed in the thermostats in test-tubes and after reaching the desired temperatures were poured on the enzyme solutions at 15 second intervals. The enzyme solutions were then placed in the thermostats and allowed to remain for 30 minutes. At the end of this time the starch solutions previously brought to the same temperatures were added to the enzyme-water mixtures at 15 second intervals and in the same order as the water-salt solutions had been added. The enzyme was allowed to act upon the starch for 30 minutes. Fehling's solution was then added and the rest of the procedure as outlined above was carried out. The method of overlapping experiments from day to day was followed here in order to guard against any deterioration of the enzyme or other possible source of error. The 30 minute period of heating without substrate was, at the higher temperatures, shortened to 15, 10, and 5 minutes in order to follow the rapid rate of destruction of the enzyme which was found to occur.

RESULTS.

As this investigation covered a wide range of temperatures it was necessary to increase the quantity of enzyme used, at 20° and 30°, in order to obtain enough cuprous oxide to weigh, while at the higher temperatures the activity of the enzymes was so increased that much smaller amounts were sufficient. In order to have a basis for the comparison of results and the plotting of curves it was decided to calculate the amounts of maltose formed by a fixed quantity of enzyme. The amount of enzyme chosen, which appeared to yield the most satisfactory values, was taken as 0.1 mg. of the dry pancreatin or of the dry matter of the malt extract. The data shown in Tables I and II were obtained in this way and give the average amount of maltose per 0.1 mg. of pancreatin or of the solids of the malt extract for each temperature. The values given under the headings "Without previous heating" in Tables I and II show the influence of temperature upon the activities of pancreatic and malt amylases in the hydrolysis of starch when acting upon the standard 1 per cent starch mixtures described above for 30 minutes at temperatures ranging from 20° to 70°C. The other data given in Tables I and II show the influence upon the enzymic activities of heating portions of the enzyme solutions in the absence of substrate in 50 cc. of the water-salt solutions described above for 30 minutes at each of the temperatures before allowing them to act upon the substrates at those temperatures. As in the latter experiments the enzyme is rapidly inactivated at

the higher temperatures it was necessary to decrease the time of preliminary heating of the enzyme in the absence of substrate.

TABLE I.
Experiments with Pancreatin.

(Average of all results calculated to 0.1 mg. of enzyme.)

Temperature	20°	30°	40°	50°	60°	70°
Without previous heating.						
Cu ₂ O, mg.....	22.9	48.7	87.1	123.7	95.8	5.7
Maltose, mg.....	18.3	39.1	70.5	100.2	77.5	4.5
After 30 min. heating.						
Cu ₂ O, mg.....	23.3	48.9	68.8	0.0		
Maltose, mg.....	18.7	39.4	55.7	0.0		
After 15 min. heating.						
Cu ₂ O, mg.....			72.8	0.0		
Maltose, mg.....			58.8	0.0		
After 5 min. heating.						
Cu ₂ O, mg.....				2.0		
Maltose, mg.....				1.6		

TABLE II.
Experiments with Malt Amylase.

(Average of all results calculated to 0.1 mg. of enzyme.)

Temperature.....	20°	30°	40°	50°	60°	70°
Without previous heating.						
Cu ₂ O, mg.....	4.5	8.4	13.6	17.9	13.7	0.7
Maltose, mg.....	3.6	6.7	10.9	14.4	11.0	0.6
After 30 min. heating.						
Cu ₂ O, mg.....	4.4	7.5	10.6	13.5	0.5	0.0
Maltose, mg.....	3.5	6.0	8.5	10.8	0.4	0.0
After 15 min. heating.						
Cu ₂ O, mg.....				14.3	2.0	
Maltose, mg.....				11.5	1.6	
After 10 min. heating.						
Cu ₂ O, mg.....					3.6	
Maltose, mg.....					3.0	
After 5 min. heating.						
Cu ₂ O, mg.....					6.8	
Maltose, mg.....					5.5	

This time was progressively decreased from 30 minutes to 15, 10, and 5 minutes. With these progressively smaller times

the rates of destruction at the lower temperatures became unmeasurable.

From the data in these tables the rate of destruction of pancreatic and malt amylases under the conditions of these experi-

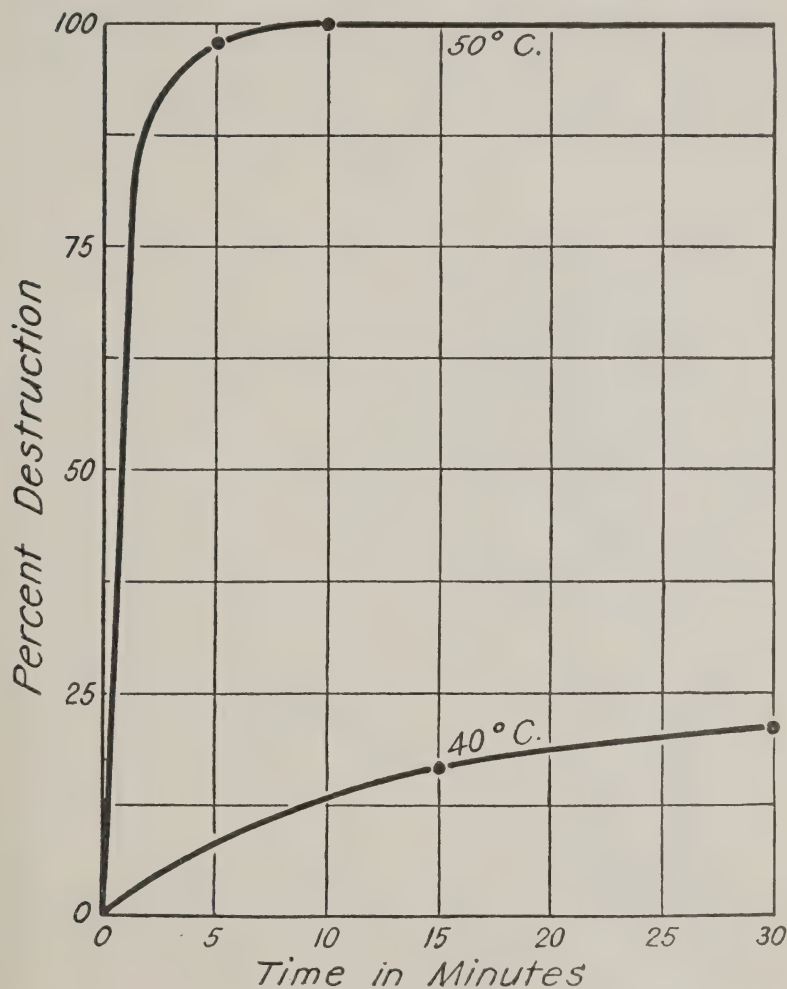


FIG. 1. The upper curve shows the rate of destruction (inactivation) of pancreatic amylase when heated in water-salt solution at 50° previous to acting on starch. The lower curve shows the rate of inactivation under the same conditions at 40°.

ments as shown in Figs. 1 and 2 were calculated as follows: The amount of sugar produced in the hydrolysis of the starch by the enzyme solutions which had not had previous heating at a given temperature was called 100 per cent. That produced after the

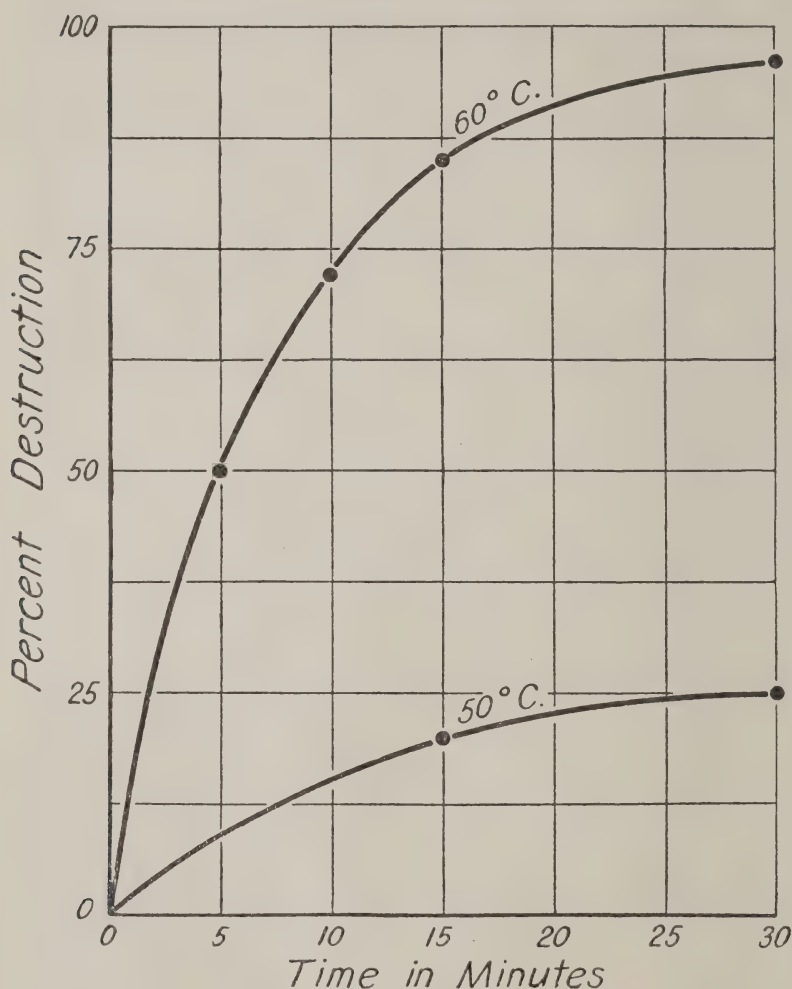


FIG. 2. The upper curve shows the rate of destruction (inactivation) of malt amylase when heated in water-salt solution at 50° previous to acting on starch. The lower curve shows the rate of inactivation under the same conditions at 40°.

different times of preliminary heating was calculated to per cent and subtracted from 100. The curves obtained from these values therefore represent the relative velocity of inactivation of these enzymes under these experimental conditions. At 50° the pancreatic amylase is about 97 per cent inactivated in 5 minutes while the malt amylase has lost only 25 per cent of its original activity after $\frac{1}{2}$ hour. At 60°, which is much above the point of complete inactivation of pancreatic amylase, the malt amylase retains a slight activity even after 30 minutes.

DISCUSSION.

It is of interest to find that increasing temperature influences the hydrolytic activities of both these enzymes in a similar manner. The values showing the activity of each obtained in the direct tests of hydrolysis at each temperature rise to a maximum at 50° with both enzymes. Evidently the increase of chemical velocity is greater up to 50°, than the inactivation of the enzymes. Beyond 50°, however, the rate of inactivation under the conditions of these experiments increases to such an extent that it becomes the predominating factor and thus the values drop very rapidly.

The effect of increasing temperature upon the activities of the enzyme solutions when they have been held without substrate at the various temperatures previous to their action upon starch at those temperatures is also of interest.

With pancreatic amylase the activity is entirely lost upon 15 minutes heating at 50°, and at 40° the values obtained upon 15 and 30 minutes heating are much below the values obtained in the direct tests of hydrolysis of starch at that temperature. This would indicate that the rate of inactivation of this enzyme increases rapidly with time and temperature. The results with malt amylase while similar to those with pancreatic amylase are less marked and indicate that malt amylase is much more stable than pancreatic amylase. This is confirmed by unpublished data from this laboratory and work carried on by Ernström (13).

At temperatures sufficiently low where the inactivation of the enzyme plays a small part, the ratio of the sugar formed by equal amounts of enzyme at the lower temperature compared to that formed at the next higher temperature is a fair measure of the velocity of the action at these two temperatures. Thus for pan-

creatin in the interval 20–30° the ratio is 2.1 and between 30° and 40° it is 1.8, while for malt at the same points it is 1.9 and 1.6. From these results it would appear that the temperature coefficient of the hydrolysis of starch by these two enzymes is about that of inorganic reactions. These results are also in accord with the data of other investigators as cited above. The velocity of the inactivation of the enzyme is, however, much more rapid than this; pancreatin, for instance, under the conditions of these experiments is destroyed thirty times as rapidly at 50° as at 40° if we compare times of equal destruction.

Malt amylase shows a greater stability toward heat, as the maximum value obtained in the inactivation experiments (Tables I and II) lies at 50° instead of at 40° as is the case with pancreatin. It also seems that the presence of starch has less protective action with malt amylase than with pancreatic amylase. That this is due to the lower activity of the malt amylase and the probable presence of impurities that may act as protecting agents is hardly a valid assumption in the light of preliminary experiments carried out with an old preparation of pancreatin of about one-third the power of sample No. 9, which would presumably contain decomposition products which would act as protective agents. This gave identical points of maxima on both the velocity and destruction curves. It would seem that malt amylase is inherently more stable than pancreatic amylase.

There are two possible explanations.

1. The inactivation of the enzyme may be an hydrolysis. The concentration of the enzyme is at all times very low. Even with the commercial preparations worked with there is never more than 2 mg. of the enzyme preparation in the 50 cc. of water used in the experiments upon heat destruction. It was first thought that possibly the dilution was a factor of importance and a series of tests were made in which the enzyme concentration was decreased to one-half, and one-fourth, but no change in the rate of inactivation could be noticed. This was interpreted to mean that the dilution was already so high that hydrolysis was at a maximum. Due to the activity of the sample and the rapid rate of starch cleavage it was impossible to increase the concentration of the enzyme much beyond the amount used. As was pointed out the destruction of pancreatic amylase at 50° in these experiments

was much more rapid than the ordinary hydrolytic reactions which have been studied previously—Arrhenius (14).

2. If the amylases are protein in nature it is possible that their inactivation might be due to heat coagulation, which, with most proteins, occurs between 50° and 70° . Sherman and Schlesinger (15), working with purified preparations of pancreatic and malt amylases, found a faint opalescence in their solutions at 50° , with increasing coagulation up to 70° , at which point, after filtering, no further precipitation would occur, even at high temperatures. It is quite possible that the previous formation of this coagulum would, on the later addition of starch, prevent any action of the enzyme. On the other hand, since enzyme action is probably a surface reaction, in the presence of the starch the enzyme at coagulation temperatures would be precipitated on the starch molecule, and though its activity would probably be less, still the action would continue beyond the point where it could react with the substrate after a preliminary heating that brought about coagulation. This theory is in accord with the evidence shown.

From the data at hand neither theory is susceptible to proof. The work of Sherman, Walker, Caldwell, and Naylor (16, 17, 18) on the protective action of certain amino acids on the amylases supports the theory that hydrolysis plays an important rôle in the heat destruction of the enzymes, but this is entirely consistent with the view that there may also be destruction by heat coagulation.

SUMMARY.

The rates of starch hydrolysis by pancreatic and malt amylases used in the forms of good grades of commercial pancreatin and malt have been determined under certain specified conditions for the temperature range of 20° to 70°C . At temperatures below the point where destruction of the enzyme plays an important rôle the rate of hydrolysis is about doubled for every 10° rise in temperature.

The temperature and rate of destruction of these enzymes in water-salt solutions have been determined and malt amylase is found to be much more stable than pancreatic amylase, the latter being completely destroyed in 15 minutes heating at 50° , while malt amylase still shows a trace of activity after 30 minutes at

60°C.; pancreatic amylase is apparently inactivated approximately thirty times as fast at 50° as is malt amylase.

The results obtained for the rate of destruction of these enzymes show a wide divergence from those giving the rates of destruction of vitamins B (19) and C (20), and make it appear doubtful that any advantage is to be gained by classing vitamins as enzymes as has sometimes been suggested.

The results obtained support the view that the heat destruction of the enzyme may be a process of the nature of the coagulation of a protein, probably accompanied by partial hydrolysis also.

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THE OPTICAL ACTIVITY OF CYSTINE.*

By JAMES C. ANDREWS.

(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.)

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Such widely differing figures have been reported for the optical activity of cystine that it is a matter of considerable doubt as to whether the different figures represent different degrees of racemization or different conditions under which the determination was made. For example, values for specific rotation as low as -200° were obtained by Gaskell (1) while Rothera (2) reported values above -250° and most of the available data range between. Moreover, Hoffman and Gortner (3) have shown that active cystine is easily racemized by heating in 20 per cent hydrochloric acid, conditions well duplicated in the ordinary method of its preparation. It was the purpose of this investigation, therefore, to study the reasons for these variations and so to standardize conditions that values from different samples of cystine shall accurately indicate their relative degrees of racemization or, the degree of racemization being constant, the relative concentration of cystine.

The insolubility of isoelectric cystine necessitates its being dissolved as either an acid or an alkali salt, but only its salts with acids are sufficiently stable to permit accurate study. Such salts hydrolyze with great ease and a large excess of acid is necessary. Possibilities of a number of reactions, therefore, present themselves and all of these may influence the composition of the asymmetric molecule and, as a result, its optical activity. For example, we may have simple dissociation of the cystine salt, and, in addition, a variety of reactions resulting from the

* An abstract of this paper was presented before the December, 1924, meeting of the American Society of Biological Chemists at Washington. D. C.

tendency of cystine or its salts to form addition compounds with either the excess of acid or water. These compounds are of the oxonium type discussed in a series of papers by Kendall (4, 5) and his collaborators and the extent of their formation is a function of the discrepancy in acidity or basicity between the cystine compound and the acid present in excess. In addition the extent of hydration of all the ions present may conceivably influence the effect of the active body on polarized light through the mechanisms to be discussed below.

EXPERIMENTAL.

A carefully mixed sample of pure cystine, analyzing 11.66 per cent of N by Kjeldahl, was used. All measurements were made in a Schmidt and Haensch polariscope, using white light and a monochromometer, and all data here recorded were taken with yellow light. A 4 dm. tube was used and with any one sample, successive readings were taken until not less than five or six checked within 0.02° . In most cases, except with the more concentrated solutions, the readings checked to the same 0.01° . All readings were taken at $29.0 \pm 0.2^\circ\text{C}$. The use of this temperature was dictated by the conditions prevailing in the room in which the polariscope was kept. The maximum temperature variation allowed while readings were taken corresponded to a negligible variation in the value of $[\alpha]_D$.

The experimental procedure used, unless otherwise noted, was as follows: 2 gm. samples of cystine were dissolved in a small amount of the acid. The solution was then made up to 100 cc. with the same acid. After the specific rotation was determined two series of dilutions were made, one with the same acid and the other with water or with a salt solution. In this way the series of acid dilutions offered decreasing cystine concentrations at practically constant pH whereas the water or salt dilutions preserved the same ratio between total acid and cystine. The use and effect of specific salt dilutions will be discussed below. Each successive dilution reduced the cystine concentration to 50 per cent of its previous value. These series were continued until the final concentration was 0.0625 gm. of cystine per 100 cc. of solution. At this concentration a difference

of 0.01° in the reading of the polariscope corresponds to a variation of 4 units in the value of $[\alpha]_D$ and further dilution was considered impracticable. Blank determinations on all diluting fluids gave negligible corrections.

The choice of acids was necessarily very limited. A large number were tried but only those discussed below dissolved enough cystine to be practicable.

The absence of mutarotation was evidenced by the fact that, once the tube has been brought to constant temperature, the reading remained constant.

Optical activity is expressed in terms of specific rotation, $[\alpha]_D$. In all cases the usual formula is used

$$[\alpha]_D = \frac{100a}{lc}$$

where a = the angle measured, l = the length of the polariscope tube in decimeters, and c , the concentration of cystine in gm. per 100 cc. of solution.

EXPERIMENTAL DATA.

Hydrochloric Acid Series.

The standard (2.000 gm.) sample of cystine was dissolved in hydrochloric acid solutions ranging in concentration from 0.5 to 2.5 M and the two series of dilutions with acid and water were made. (See Table I and Fig. 1.)

The data in Table I are typical. With most acids the curves representing the effect of water dilution (constant ratio of cystine: acid) and acid dilution (constant pH) diverge in the same way as do those of hydrochloric acid. It should be noted that in acid dilution curves, a slight maximum value of $[\alpha]_D$ becomes evident at 1.5 M HCl and above, while a gradual slope is obtained with 0.5 M HCl so that the most constant values of $[\alpha]_D$ are those resulting from acid dilution of the cystine hydrochloride from 2.0 to 0.5 gm. in 1.0 M HCl. The significance of these curves will be more fully discussed below. It is also interesting to observe that at high dilutions, values of $[\alpha]_D$ of from -200° to -270° were obtained. Aside from the possibility of various degrees of racemization, changes in the conditions of the determination, such as are de-

scribed above, amply account for the wide range of values previously recorded in the literature.¹

In order to recheck the divergence at low concentrations of cystine a further series of determinations was made in which a stock solution was used containing 0.5 gm. of cystine per 100

TABLE I.
 $-\alpha_D^{29}$ of Solutions of Cystine Hydrochloride. Effect of Variations in Concentration of Cystine Salt and of Hydrochloric Acid.

<i>C</i> cystine per 100 cc. solution.	$-\alpha_D^{29}$ in 0.5 M HCl.		$-\alpha_D^{29}$ in 1.0 M HCl.		$-\alpha_D^{29}$ in 1.5 M HCl.		$-\alpha_D^{29}$ in 2.0 M HCl.		$-\alpha_D^{29}$ in 2.5 M HCl.	
	Acid dilu- tion.	H ₂ O dilu- tion.	Acid dilu- tion.	H ₂ O dilu- tion.	Acid dilu- tion.	H ₂ O dilu- tion.	Acid dilu- tion.	H ₂ O dilu- tion.	Acid dilu- tion.	H ₂ O dilu- tion.
<i>gm.</i>										
2.00	222.4	222.4	215.5	215.5	210.0	210.0	207.7	207.7	203.0	203.0
1.00	221.8	231.0	215.5	223.8	211.0	220.0	208.7	216.0	206.7	215.7
0.50	218.5	240.5	215.0	233.5	209.5	228.0	209.0	223.0	207.5	228.0
0.25	217.0	251.0	214.0	243.0	209.0	237.0	208.0	233.0	207.0	236.0
0.125	210	260	208	254	208	248	204	242	204	242
0.0625	204	270	200	268	204	256	200	252	200	252

TABLE II.
 $-\alpha_D^{29}$ of Solutions of Cystine Hydrochloride, $C = 0.05$ Gm. per 100 Cc.

Normality of HCl.	$-\alpha_D^{29}$
0.0125	265
0.0526	237
0.1128	230
0.1930	225
0.4625	205
0.9125	195
1.3625	192
1.8125	190
2.2625	185

cc. of 0.125 M HCl. Portions of this solution were diluted ten times with water and with varying concentrations of HCl and

¹ Similar variations were obtained with the sulfuric and oxalic acids, although the actual values are different. 2.00 gm. of cystine per 100 cc. in 1.00 M sulfuric and oxalic acids gave, respectively, values for $[\alpha]_D$ of 189.6 and 221.5. Attempts to run a series of determinations with cystine nitrate proved impractical because of gradual oxidation of the cystine.

rotations were taken. (See Table II and Fig. 2.) These figures completely substantiate the results previously obtained with high dilutions of cystine.

Phosphoric Acid Series.

Owing to the lower degree of ionization of phosphoric acid higher concentrations were necessary than in the experiments previously described. The data recorded in Table III and illustrated in Fig. 3 were obtained with 2.00 and 4.00 M solutions.

The remarkable results obtained from the acid dilutions at low cystine concentrations led at once to careful rechecking, but in both cases the form of the curve was fully substantiated.

Picric Acid Series.

Picric acid, although too insoluble to permit the investigation of a complete series, was nevertheless studied. 100 cc. of a saturated solution (0.0505 M) dissolved about 0.15 gm. of cystine. From this solution, water and acid dilutions were then made in the usual way. (See Table IV and Fig. 4.) The constant values obtained by diluting with water make this system distinct from any other studied.

Trichloroacetic Acid Series.

Experiments with this acid were confined to 1.00 and 2.00 M solutions. The data, recorded in Table V and Fig. 5, introduce a new condition in which the acid curve retains its normal form while dilution with water causes the specific rotation to fall to a minimum value and then rise in the usual manner.

Sulfosalicylic Acid Series.

Sulfosalicylic acid ($\text{C}_6\text{H}_3(\text{OH})(\text{SO}_3\text{H})(\text{COOH})$) (6) was selected as an example of an organic acid with a degree of ionization comparable to that of hydrochloric acid. 0.5 and 2.00 M solutions were used. These data presented no new features except that change in concentration of the acid was almost entirely without effect on the optical activity. 2 gm. of cystine per 100 cc. of 0.5 and 2.00 M acid gave, respectively, values for $[\alpha]_{\text{D}}^{29}$ of 221.0 and 221.5.

TABLE III.

$-\alpha_D^{29}$ of Solutions of Cystine Phosphate. Effect of Variations in Concentrations of Cystine Salt and of Phosphoric Acid.

C cystine per 100 cc. solution.	$-\alpha_D^{29}$ in 2.0 M H_3PO_4 .		$-\alpha_D^{29}$ in 4.0 M H_3PO_4 .	
	Acid dilution.	H ₂ O dilution.	Acid dilution.	H ₂ O dilution.
gm.				
2.0	203.4	203.4	195.9	195.9
1.0	205.0	217.7	194.3	204.5
0.5	205.0	228.5	193.5	215.5
0.25	204.0	239.0	193.0	223.0
0.125	204	250	192	232
0.0625	204	256	192	248

TABLE IV.

$-\alpha_D^{29}$ of Solutions of Cystine Picrate. Effect of Variations in Concentration of Cystine Salt and of Picric Acid.

C cystine per 100 cc. solution.	$-\alpha_D^{29}$ in 0.0505 M picric acid.	
	Acid dilution.	H ₂ O dilution.
gm.		
0.15	207	207
0.075	203	206
0.0375	200	207

TABLE V.

$-\alpha_D^{29}$ of Solutions of Cystine Trichloroacetate. Effect of Variations in Concentration of Cystine and Trichloroacetic Acid.

C cystine per 100 cc. solution.	$-\alpha_D^{29}$ in 1.00 M trichloroacetic acid.		$-\alpha_D^{29}$ in 2.00 M trichloroacetic acid.	
	Acid dilution.	H ₂ O dilution.	Acid dilution.	H ₂ O dilution.
gm.				
2.00	232.0	232.0	247.1	247.1
1.00	231.2	227.0	247.5	232.2
0.50	231.0	228.5	248.0	227.0
0.25	226.0	234.0	245.0	225.0
0.125	216.0	240	238	230
0.0625	208	248	236	232

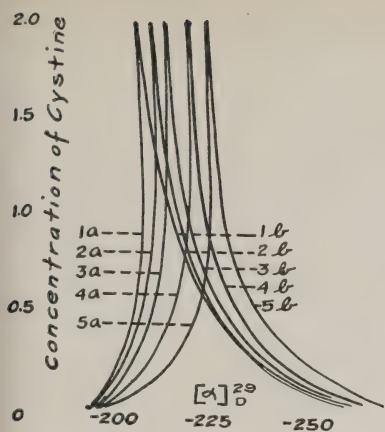


FIG. 1.

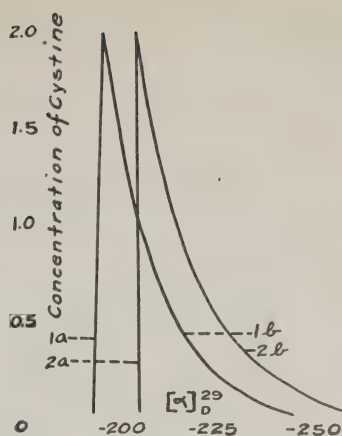


FIG. 3.

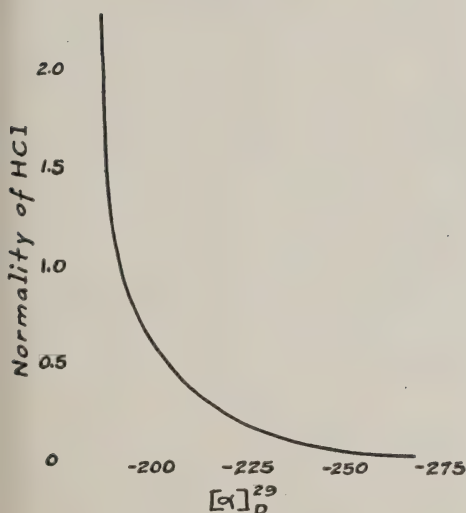


FIG. 2.

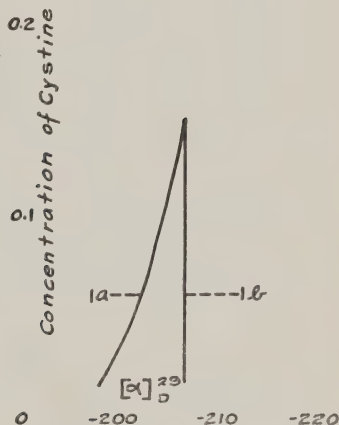


FIG. 4.

FIG. 1. Specific rotation of cystine hydrochloride.

1 a = 2.5 M HCl, acid dilution; 1 b = 2.5 M HCl, water dilution.

2 a = 2.0 M HCl, acid dilution; 2 b = 2.0 M HCl, water dilution.

3 a = 1.5 M HCl, acid dilution; 3 b = 1.5 M HCl, water dilution.

4 a = 1.0 M HCl, acid dilution; 4 b = 1.0 M HCl, water dilution.

5 a = 0.5 M HCl, acid dilution; 5 b = 0.5 M HCl, water dilution.

FIG. 2. Specific rotation of cystine hydrochloride at high dilution (0.05 gm. per 100 cc.). Effect of HCl concentration on specific rotation.

FIG. 3. Specific rotation of cystine phosphate.

1 a = 4.0 M H₃PO₄, acid dilution; 1 b = 4.0 M H₃PO₄, water dilution.

2 a = 2.0 M H₃PO₄, acid dilution; 2 b = 2.0 M H₃PO₄, water dilution.

FIG. 4. Specific rotation of cystine picrate.

1 a = acid dilution series; 1 b = water dilution series.

*Salt Series.**2.50 M Hydrochloric Acid.*

In some series, successive dilutions were made with solutions of a salt instead of the free acid, thereby diminishing the acidity while maintaining the same concentration of the negative radical. It seemed of considerable interest to ascertain whether the important factor in determining the form and position of dilution curves was the total osmotic concentration, the negative ion concentration, the pH, or some totally different factor. In the first and second cases, the curve resulting from dilution with an equivalent concentration of salt should nearly approximate the acid dilution curve; in case the pH of the solution was the controlling factor the salt dilution curve should closely approach that produced by water dilution.

A solution of 2.0 gm. of cystine in 100 cc. of 2.500 M HCl was made up and aliquots were diluted in the usual way with 2.50 M NaCl, 0.50 M NaCl, and also, for further comparison, with 0.50 M sodium sulfosalicylate solution. (See Table VI and Fig. 6.) In order to facilitate comparison with the new curves, those previously described and recorded for 2.50 M HCl are again included. The depressing effect of NaCl on the position of the water dilution curve (on $[\alpha]_D$) is very evident. Sodium sulfosalicylate had a much smaller effect.

Trichloroacetic Acid.

Solutions of cystine in 1.00 and 2.00 M trichloroacetic acid were diluted with 1.00 and 2.00 M solutions of sodium trichloroacetate² and with 1.00 and 2.00 M sodium chloride. (See Table VII and

² The curves of dilution with sodium trichloroacetate are subject to a special source of error because of the instability of this salt, even at room temperatures. Sodium trichloroacetate hydrolyzes very easily into chloroform and sodium bicarbonate; the action goes rapidly to completion at temperatures near the boiling point, and the only practicable method of employing the salt is to make and use it in solution immediately, keeping the solution as cold as possible. The salt solution was made by mixing equal volumes of carefully standardized NaOH and CCl_3COOH . The solutions were cooled in an ice bath and then mixed with rapid stirring. Even with these precautions, the solution smelled of chloroform before the series was completed.

Figs. 7 and 8.) There is a consistent tendency on the part of sodium trichloroacetate to raise the low values of $[\alpha]_D$ which result from water dilution. This is in decided contrast to the opposite effect exhibited by sodium chloride.

TABLE VI.

$-\alpha_D^{29}$ of Solutions of Cystine Hydrochloride. Effect of Variations in Concentrations of Cystine Salt and of Sodium Chloride and Sodium Sulfosalicylate.

C cystine per 100 cc. solution.	$-\alpha_D^{29}$ in 2.50 M HCl.				
	Acid dilution.	H ₂ O dilution.	2.50 M NaCl dilution.	0.50 M NaCl dilution.	0.50 M Na sulfosalicylate dilution.
gm.					
2.0	203.0	203.0	203.0	203.0	203.0
1.0	206.7	215.7	201.0	208.5	213.0
0.5	207.5	228.0	198.0	214.0	224.0
0.25	207.0	236.0	199.0	221.0	233.0
0.125	204	242	203.0	227	242
0.0625	200	252	208	236	256

TABLE VII.

$-\alpha_D$ of Solutions of Cystine Trichloroacetate. Effect of Variations in Concentration of Cystine Salt and of Sodium Trichloroacetate and Sodium Chloride.

C cystine per 100 cc. solution.	$-\alpha_D$ in 1.00 M trichloroacetic acid.			$-\alpha_D$ in 2.00 M trichloroacetic acid.				
	Acid dilution.	H ₂ O dilution.	Salt dilution.	Acid dilution.	H ₂ O dilution.	Salt dilution.	1.00 M NaCl dilution.	2.00 M NaCl dilution.
gm.								
2.00	232.0	232.0	232.0	247.1	247.1	247.1	247.1	247.1
1.00	231.2	227.0	232.0	247.5	232.2	253.0	228.8	224.8
0.50	231.0	228.5	233.0	248.0	227.0	253.6	217.8	210.0
0.25	226.0	234.0	238.0	245.0	225.0	255.6	215.0	205.0
0.125	216	240	244.0	238	230	258	216	206
0.0625	208	248	260	236	232	266	225	218

Sulfosalicylic Acid.

The dilution of a solution of cystine sulfosalicylate with sodium sulfosalicylate and sodium chloride solutions gave results in keeping with what might have been expected from the previous behav-

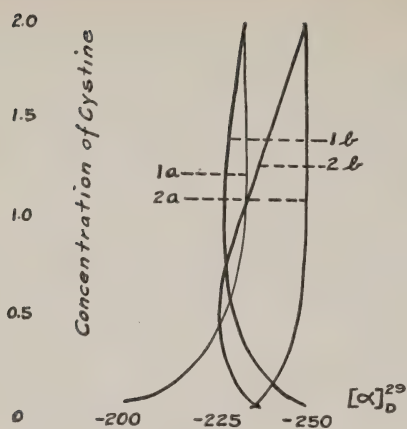


FIG. 5.

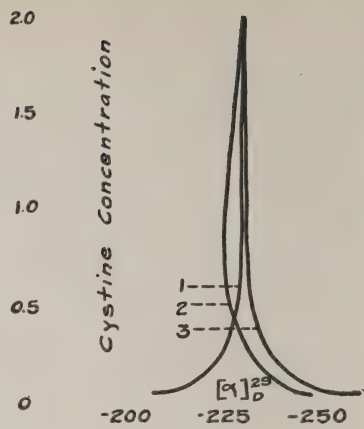


FIG. 7.

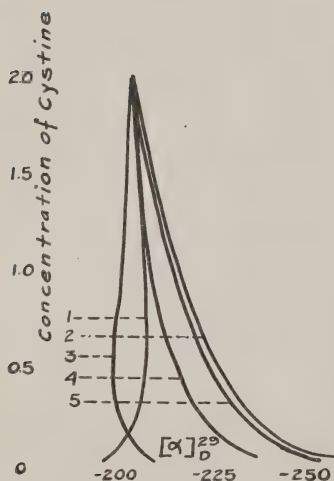


FIG. 6.

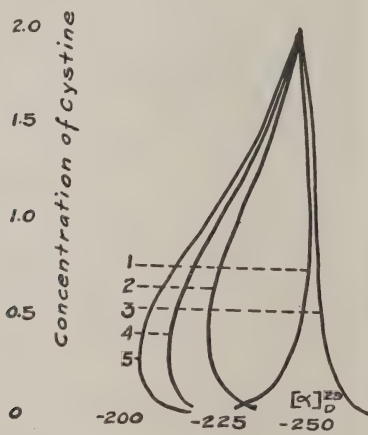


FIG. 8.

FIG. 5. Specific rotation of cystine trichloroacetate.

1 a = 1.0 M CCl_3COOH , acid dilution; 1 b = 1.0 M CCl_3COOH , water dilution; 2 a = 2.0 M CCl_3COOH , acid dilution; 2 b = 2.0 M CCl_3COOH , water dilution.

FIG. 6. Specific rotation of cystine hydrochloride diluted as follows: (1) 2.5 M HCl dilution; (2) water dilution; (3) 2.5 M NaCl dilution; (4) 0.5 M NaCl dilution; and (5) 0.5 M sodium sulfosalicylate dilution.

FIG. 7. Specific rotation of cystine trichloroacetate diluted as follows: (1) 1.0 M CCl_3COOH dilution; (2) water dilution; and (3) 1.0 M CCl_3COONa dilution.

FIG. 8. Specific rotation of cystine trichloroacetate diluted as follows: (1) 2.0 M CCl_3COOH dilution; (2) water dilution; (3) 2.0 M CCl_3COONa dilution; (4) 1.0 M NaCl dilution; and (5) 2.0 M NaCl dilution.

ior of these salts. Sodium sulfosalicylate slightly raised the values of $[\alpha]_D$ obtained from the water dilution of cystine sulfosalicylate while sodium chloride lowered these values.

DISCUSSION.

Examination of these results reveals several fairly general characteristics.

1. At low concentrations of cystine (less than about 0.2 gm. per 100 cc. or 0.0083 molar) the value of $[\alpha]_D$ seems to depend chiefly on the pH of the solution, low pH values giving low values for $[\alpha]_D$.

2. At higher concentrations of cystine than 0.2 gm. per 100 cc. the effect of various ions is almost entirely specific and peculiar to the particular ions present.

3. For any given acid, dilution with a constant concentration of that acid (at practically constant pH) results in practically constant values for $[\alpha]_D$. That this constancy is at least partially a result of constant hydrogen ion concentration is indicated by the fact that substitution of an equimolar concentration of the sodium salt for the acid results in marked changes in the form of the dilution curves.

The curves resulting from water dilution show the form characteristic of dissociation reactions and probably represent, in the main, the dissociation of the cystine salt into its ions. To what extent this salt has also combined with the excess acid to form the oxonium compound and to what extent the dissociation of this compound influences the form of the water dilution curve seems impossible to say from the present data. Hädrich (7) determined the effect of dilution on the optical activities of the salts of several alkaloids with strong acids and showed that the resulting curves in most cases, followed the course predicted on the basis of ionic dissociation. It was unnecessary, however, for Hädrich to employ the large excesses of acid that were required in the present work and he was not, therefore, led to an investigation of the effect of varying amounts of other electrolytes. In the case of a number of other investigators, notably Rimbach (8), similar attempts to establish the exclusive influence of simple ionization on the rotation of active electrolytes were unsuccessful.

Particular interest attaches the few points determined for the

picric acid series. If we assume the usual form of the water dilution curves to be the result of electrolytic dissociation of the cystine salt we must conclude that the evidence here points to complete lack of dissociation under the conditions of the experiment. This is noteworthy in view of the frequent use of picric acid as a precipitant of organic bases.

In connection with these data, the work of Patterson (9) on the effect of internal pressure on optical activity is of interest. Patterson assumes that the shape of an asymmetric molecule is influenced by the internal pressure of the solution and that this shape, or some function of it, is directly responsible for its specific rotation. His data show, moreover, the particularly great influence of internal pressure at high dilutions of the active solute.

In the present work, similar influences undoubtedly come into play. The substance studied differs from those of Patterson's work in being of electrolytic nature³ and in the present work changes in internal pressure are effected chiefly by changing the ionic content of the solution. The connection between electrolyte concentrations and the molecular volume of the active solute (a direct function of internal pressure) is obvious. It is, therefore, not surprising that the ions, with their different degrees of hydration, cause the highly specific effects observed in the various salt dilution curves. It does not seem profitable to do more than indicate the direction in which the explanation for these data lies.

The above experiments permit the standardization of conditions for the determination of the optical activity of cystine. The very erratic values for $[\alpha]_D$ of cystine which appear in the literature may be amply explained by the variety of conditions employed, although frequently no description of these has been included. The necessity for such particularization is shown by the data in the present paper. Inspection of the data presented above shows that the most constant and easily duplicable conditions for the determination of $[\alpha]_D$ of cystine are to be obtained by the use of HCl of constant concentration (preferably 1.00 M), containing 1.0 gm. of cystine per 100 cc. of solution. A glance

³ Patterson worked chiefly with ethyl tartrate in water and various organic solvents.

at Table I shows that with this concentration of acid, the effect of variation in the proportion of cystine used is very slight. Convenience will, in many cases, dictate the use of a lower temperature than that used in this work. Comparison of readings, taken at both 20° and 29°C., has showed an average temperature coefficient of $-1.7^{\circ} [\alpha]_D$ per 1°C. over this range of temperature.

SUMMARY.

The optical activity of cystine in the form of a variety of its salts with strong acids was studied. The effect of changes in such conditions as the concentration of cystine, of excess acid, and of added salts was determined. Various mechanisms influencing the optical activity of cystine have been discussed and a probable method by which these influences are brought into play has been suggested. This method involves the effect of the varying degrees of hydration of different ions on the internal pressure of the solution and the consequent effect on the shape of the asymmetric molecule. Standard conditions for the determination of the optical activity of cystine have been outlined.

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THE OXIDATION OF CYSTINE.

By JAMES C. ANDREWS.

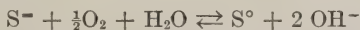
(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.)

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In 1909 Mathews and Walker¹ studying the oxidation of cystine, concluded that this compound in alkaline solution is oxidized by atmospheric oxygen. While this conclusion has been generally accepted, the experiments described below furnish evidence that cystine is not directly oxidized and that the oxygen consumption results from the oxidation of sulfide ion formed from cystine by the action of the alkali.

The method used by Mathews and Walker to measure the oxidation of cystine consisted in keeping the alkaline cystine solution in contact with air in a flask provided with a mercury manometer, the rate of absorption of oxygen by the solution being measured by the diminishing pressure in the flask. Different cystine solutions showed varying speeds of oxygen absorption, depending on the purity of the sample, the concentration of the alkali, and the presence of such catalysts as KCN, Fe⁺⁺⁺, etc. From these experiments these authors concluded that cystine oxidized spontaneously in alkaline solution, although they made no attempt to determine the products of oxidation.

The precipitation of "sulfide sulfur" from alkaline cystine solutions has given, in the hands of different workers, yields of sulfur varying from about 52 to 83 per cent of the total sulfur, depending on the conditions of the experiment. The extent of sulfide formation varies within a wide range. However, in the absence of any heavy metal to precipitate the sulfide as fast as it is formed, its oxidation may easily take place according to the well known reaction



¹ Mathews, A. P., and Walker, S., *J. Biol. Chem.*, 1909, vi, 289.

The free sulfur, dissolving in excess sulfide to form the polysulfide, is undoubtedly responsible for the yellow color of these solutions and for the precipitation of free sulfur on acidification.

In order to compare the rate of oxidation of sodium sulfide with that observed by Mathews and Walker with cystine, solutions of sodium sulfide containing 1 gm. of pure $\text{Na}_2\text{S} \cdot 9 \text{H}_2\text{O}$ in 50 cc. each of water, of 2.0 M NaOH, and of 4.0 M NaOH were used. The apparatus was arranged as described by Mathews and Walker and the change in pressure read from time to time. After 20 hours the reduction in pressure in the three flasks (in the order listed above) was 112, 115, and 71 mm., a speed of absorption comparable with that obtained by Mathews and Walker with pure cystine.

Other experiments of this type gave similar values. These results emphasize the fact that the well known tendency for sodium sulfide to absorb oxygen and form the polysulfide can produce absorption results comparable to those obtained with alkaline cystine solution.

However, more direct evidence that the cystine itself is unaffected is desirable. The optical activity of alkaline solutions of cystine affords a valuable criterion of the oxidation of this compound. Considering the very great responsiveness of optical activity to change in composition it seems inconceivable that the oxidation of cystine should not be accompanied by profound changes in activity. Samples of cystine, which in acid solution give $[\alpha]_D$ values of over 200, usually give, in alkaline solutions, values of about 75 or less. These drop very quickly, probably because of rapid racemization together with some decomposition in the strong alkali. This drop, however, is not due to any oxidation of the cystine, as the following experiments indicate.

A solution of 2.00 gm. of pure cystine per 100 cc. of 4.00 M NaOH was divided into two portions immediately after its preparation. Pure nitrogen was bubbled through one for about $1\frac{1}{2}$ hours and air through the other for the same period. The optical rotation of each solution was then taken three successive times at 15 minute intervals with the following results.

Time read.	$[\alpha]_D^{29}$ of the solution in	
	Air.	Nitrogen.
At once.	63.0	62.8
15 min. later.	59.4	59.4
30 " "	56.2	56.0

Because of the gradual formation of polysulfide, the solution exposed to air became darker and more quickly rendered accurate readings difficult, but the duplication of optical activity is quite apparent.

To obtain still more conclusive results, the following experiment was then made.

Two 1.000 gm. samples of the same lot of cystine as used above were each dissolved in 25.0 cc. of 4.00 M NaOH. Pure oxygen was passed through one for 30 minutes and pure nitrogen through the other. At the end of that time, 50.0 cc. of 4.00 M HCl were added to each solution plus enough water to make each volume to 100.00 cc. Each solution, therefore, contained 1.000 gm. of cystine per 100 cc. in 1.0 M HCl. Each was then filtered into a polariscope tube and the specific rotations of the oxygen and the nitrogen samples were found to be 166.7 and 166.5, respectively. The cystine exposed to oxygen showed no more change than did the sample exposed only to nitrogen, where oxidation was impossible. This sample of cystine would have given a value of about 207 had it not been partially racemized and decomposed by the alkali.

In a further experiment, the above conditions were duplicated with the exception that to each of the alkaline solutions of cystine there were added 10 cc. of a half saturated lead acetate solution. The two gases were then passed through for 2 hours each, during which time a small precipitate of lead sulfide formed in each flask. Each solution was then acidified exactly as before, filtered, and polariscope readings were taken. In this case there was no detectable difference between the two. The value for $[\alpha]_D^{25}$ for each was 152.5.

In another case, the conditions of the last experiment were duplicated exactly except that the oxygen and nitrogen were bubbled into the flasks for 3 days before the solutions were acidified and rotations were taken. The specific rotation for the oxygen and nitrogen samples were then, respectively, 16.0 and 15.8. Even when the S^{2-} was kept at a minimum with Pb^{++} and the oxygen was given a longer time to act, the decomposition of the cystine is as great in one case as in the other.

Further evidence seems hardly necessary to show that, even though oxygen is absorbed, cystine in alkaline solution is not directly oxidized. There are other important changes which result from the introduction of oxygen into such a solution but

these concern entirely different equilibria, which will be discussed in a future paper. The conclusion seems inevitable that the substance of which Mathews and Walker measured the rate of oxidation was sulfide ion rather than cystine.

SUMMARY.

A study of the behavior of cystine in alkaline solution leads to the conclusion that it is not directly oxidized by atmospheric oxygen and that the observed absorption of oxygen is due to oxidation of sulfide ion.

STUDIES OF THE METABOLISM OF WOMEN.

I. VARIATIONS IN THE FASTING BLOOD SUGAR LEVEL AND IN SUGAR TOLERANCE IN RELATION TO THE MENSTRUAL CYCLE.

BY RUTH OKEY AND ELDA I. ROBB.

(From the Laboratory of Household Science, University of California, Berkeley.)

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INTRODUCTION.

The literature on the general question of the significance of the menstrual phenomenon has, recently, been so well summarized by Corner (1) and by Marshall (2) that any attempt at further review seems out of place here. That the present state of our knowledge with regard to the biochemical and metabolic changes involved in the monthly cycle in women is eminently unsatisfactory must, however, be conceded. In fact, until we have realized the extent of the difficulties encountered by the investigator in a field so hedged about by social and religious conventions, we are tempted to conclude that almost nothing has been achieved toward a rational understanding of these aspects of the phenomenon.

It has been estimated that the time during which the average woman is menstruating aggregates a total of 7 years (3). For from 65 to 75 per cent of all civilized women, some part, at least, of this period, is marked by pain and a greater or lesser degree of incapacitation for work. It must appear, moreover, that the best prospect for the achievement of an ultimately satisfactory interpretation of this practically important phenomenon rests upon the accumulation of data from chemical and physiological as well as anatomical and histological measurements. The results of the series of studies reported in this paper and those which are to follow are therefore offered in the hope that they may be of some value, even if they serve only as temporary units in the building of a more satisfactory understanding of the cyclic changes in the life processes of women.

LITERATURE.

Carbohydrate Metabolism.

That the metabolism of carbohydrates may be affected, directly or indirectly, by the activities of organs of internal secretion other than the pancreas, is generally granted. The rôle of the individual glands and their influence, one upon the other is, however, very imperfectly understood. Hypersecretion of the suprarenals is supposed to lead to increased mobilization of glucose from the glycogen storehouses, with an accompanying high level of blood sugar, and, possibly, glycosuria.

Riddle and Honeywell (4, 5) have recently observed, in various types of pigeons, a suprarenal hypertrophy coincident with ovulation. There was an accompanying increase, reaching 20 per cent, in the blood sugar. This began approximately 108 hours after the ovulation of the first egg of a pair, was maintained at a maximum level throughout the time of ovulation, and gradually decreased to the resting level within 108 hours after the ovulation of the last egg. These investigators noted that the curve of blood sugar values was essentially similar to that of suprarenal hypertrophy.

Their conception of a rhythmic variation in carbohydrate metabolism coincident with a like variation in sexual activity impresses the reader with its similarity to the idea of rhythmic variation in the nutritive processes of women expressed by Jacobi (3). The difficulty in making direct comparisons arises from the fact that while the probabilities are (1) that ovulation in women takes place from 10 days to 2 weeks before the onset of menstruation no definite time relationships between the two phenomena have, as yet, been established. Moreover, there is much evidence (6) that the activities of the ovary as a gland of internal secretion must be considered, not as a single entity, but as a complex, resulting from the balanced effects of corpus luteum, follicles, interstitial cells, etc. To some extent, also, there is evidence (7) of antagonism between certain functions of the ovaries and of the suprarenals in that atrophy of the ovaries with hypertrophy of the suprarenals sometimes results in loss of secondary sexual characteristics in women.

Very few efforts to correlate the human menstrual cycle with physiological variations in blood sugar, or in sugar tolerance, have been reported in the literature; but the reports which we do have are distinctly contradictory.

H. Kahler (8) observed a slight rise in blood sugar at the time of the onset of the menstruation, with normal values for the same individuals at the end of the period. His determinations were, however, made by the method of Bang, using finger blood, and the observed variations were so small as to fall within the limits of experimental error.

Ernst Hoffmann (9) decided, on the other hand, that values for sugar in the blood serum were lower, and that the tolerance toward galactose and levulose was increased during menstruation. He considered, on the basis of this evidence, that the influence of the ovarian hormone on carbohydrate metabolism was quite marked.

This investigator made two determinations of serum sugar for each of his subjects, 1 hour after the ingestion of a test meal; the first in the intermenstrual period, and the second during menstruation. He had fifteen cases, to four of whom he gave 100 gm. of levulose; to ten, 30 to 40 gm. of galactose; and to one, 80 gm. of lactose. Eleven showed a typical lowering of serum sugar (from 0.01 to 0.03 per cent). In two cases (one a thyroid, and one an arthritis patient), he observed no change, and in two cases (a girl of 14 listed as a "nosebleed" case, and a chlorosis patient), there were increases of 0.03 and 0.01 per cent respectively, in the serum sugar at the time of the menstrual period. It is interesting to note that eight of the eleven cases who reacted with a smaller increase in serum sugar at the time of menstruation were listed as "*gesund*," while the others were suffering from a variety of disorders.

Hoffmann also found, in rabbits, and in three of the four women whom he treated with "Luteovar, Poehl," a similarly decreased response to galactose ingestion as measured by serum sugar determinations.

The method which he used for sugar estimation was a modification of that of Bertrand, and involved the taking of a considerable amount of blood. The fact that galactose and lactose were used as the test carbohydrates leads us to wonder how far his conclusions might be modified by repetition and reinterpretation of this

work in the light of the recent contributions of Folin and Berglund (10), and of Berglund and Ni (11) to our knowledge of the mechanism of the metabolism of these sugars.

In sharp contrast to this work of Hoffmann stands that of Rosenbloom (12), who has reported a large decrease in the food tolerance of diabetic patients during menstruation. This was accompanied by glycosuria, acidosis, and a marked increase in the tendency to develop coma. One of the two patients observed died as a result of this menstrual break. The loss of food tolerance was explained as due to an increase in the size, and, presumably, in the functional capacity of thyroids, suprarenal cortex, and pituitary glands, and some alteration in the functioning of the gonads which resulted in a temporary lack of correlation of endocrine activity.

Harrop and Mosenthal (13) have also reported a case in which the sugar tolerance was markedly lessened during the menstrual period. They seem to believe that there is a possibility that menstruation in diabetic women is frequently coincident with terminal acidosis and coma.

EXPERIMENTAL.

The work reported here has been undertaken with the idea of determining, if possible, (a) whether or not there are any consistent variations in the fasting blood sugar levels in women coincident with the different phases of the monthly cycle; and (b) whether the response to the ingestion of a given amount of glucose in terms of rise in blood sugar level and urinary excretion of sugar is consistently different at different times during the month in the same individual.

Subjects.

The subjects were young women, upper division and graduate students from the classes in Nutrition and preparatory Medicine, who volunteered for this work because of interest in it, and who were willing and able to give intelligent cooperation. All of them qualified as "normal" from a clinical point of view. Health records and records of physical examinations, etc., were obtained through the courtesy of the staff of the University of California Infirmary and supplemented by questioning the individual subjects.

The three girls who constituted Group I were subjects for a detailed study of nitrogen metabolism in its relationship to the menstrual cycle, and were on weighed and analyzed diets¹ which were kept constant for each individual for at least 1 month at a time. Group II consisted of subjects who were on the ordinary diets at their respective boarding clubs. They were, however, instructed to refrain from any large variations in their usual routine of living while the experimental period lasted and, as far as could be ascertained, followed instructions. Group III, the detailed figures for which are not included in the tables, consisted of students originally expected to be in Group II, who became ill during the experimental period or whose Infirmary record did not seem to justify rating as "normal." Their blood sugar values, however, show no very marked differences from those of the other groups. Data for two men observed as controls over periods of 1 month each is likewise not given in detail, since these periods included several shorter ones on experimental diets of rather radical nature. Day to day variations in blood sugars were approximately the same as those observed in women during the intermenstrual period.

Every precaution possible with a group of student subjects was taken to eliminate the effect of disturbing factors such as excitement, fatigue, etc. It is fully recognized, however, that the life

¹ A detailed description of these diets will be given in a later paper. They were planned with the idea of studying nitrogen metabolism at different levels of protein intake.

Diet I represented the ordinary mixed diet, well balanced, but containing a comparatively small amount of protein. Meat was included, but the total protein aggregated 50 gm., with 250 gm. of carbohydrate.

Diet II was likewise well balanced, but it represented a somewhat higher level of protein intake (70 gm. of protein and 300 gm. of carbohydrate), was meat-free and contained very little purine.

Diet III contained 150 gm. of protein and 250 gm. of carbohydrate, with as little purine as could be obtained without limiting the intake to purified foodstuffs, or excluding necessary sources of vitamin.

Diet IV was designated as "low protein, purine-free." It contained approximately 20 gm. of protein, with very little purine or creatine. The protein was, however, of good quality and the diet was otherwise entirely adequate. Calories were in each case adjusted to the needs of the individual, largely through variation of the fat intake. All diets were very well tolerated, with the possible exception of Diet III in one case.

of the present day college woman lacks most of the elements of regularity which we require in the routine of existence of our laboratory animals. It has seemed, however, that the more intelligent cooperation which we have received from our student subjects has more than balanced the better control of activities possible with institutionalized patients, who are usually not only not normal, physically, but either unwilling or unable to follow instructions.

The factor of excitement can probably be considered to have been ruled out, very successfully. The young women were accustomed to act as subjects for various types of experimental work, and blood samples included in the series were, as a rule, obtained with a minimum of difficulty. The work of Foster (14) indicates, moreover, that emotional hyperglycemia with this type of subject is practically negligible, even where the taking of the sample involves considerable pain.

The effect of exercise was not so easy to eliminate. Blood samples were taken from the members of Group I immediately after the measurement of basal metabolism, consequently they represent resting values. The members of Group II came to the laboratory, however, from a greater or lesser distance, and because of class schedules, it was not usually possible to secure a rest period before taking the samples. In most cases, nevertheless, it has been impossible for us to demonstrate any appreciably greater degree of day to day variation in samples taken from the subjects immediately after coming to the laboratory than in those who had a rest period before bleeding. This is, perhaps, not so contradictory to the findings of Rakestraw (15) as it may at first appear. His increases in blood sugar level were found only after quite short periods of very violent exercise in no way comparable to the more moderate, longer sustained exertion involved in the morning procedure of dressing and coming to the laboratory. Moreover, he has shown that very little change in blood sugar level is produced by long continued exercise.

Methods.

The blood samples were taken from the median veins in the arm, mixed with powdered lithium oxalate, and the protein precipi-

tated according to the method of Folin-Wu (16). Analyses for sugar were made within the first few hours thereafter, by the improved method of Folin-Wu (17). Fresh glucose standards containing benzoic acid were made up, each week, and the old standard was analyzed against the new to check against any possible deterioration. Every care was taken, also, to eliminate day to day variations in the method of procedure.

The sugar tolerance determinations were made according to a modification of the technique of Killian (18). The glucose used was Merek's C. P. It was given in doses of 1.75 gm. per kilo of body weight, in 50 per cent solution, and without any preliminary meal. The amount of water taken at this time and subsequently during the test (usually 100 ml. per hour) was carefully measured. The possibility of blood dilution was checked by determinations of hemoglobin carried out according to the method of Cohen-Smith (19) as modified by Robscheit (20). Determinations of urine sugar were made by the method of Folin-Berglund (21). Creatine and creatinine were determined in blood and urine of the first five subjects in the tolerance series, but these determinations were afterwards abandoned as time-consuming and giving no significant results.

DISCUSSION.

Inspection of the data given in Tables I and II must lead to the conclusion that, whatever the influence of the menstrual cycle on carbohydrate metabolism, it is impossible to state positively, on the basis of "before breakfast" blood sugar determinations, that there is a uniform and consistent variation in the fasting level of blood sugar at any one phase of the monthly cycle in women. An attempt to summarize the results of a series of determinations, such as that given in Table III leads, however, to a suspicion that the time of the menstrual period may be marked by a greater degree of susceptibility to variation of blood sugar level than any other part of the cycle. The average menstrual value is slightly higher than the average intermenstrual value. If, in computing the normal average for each individual, we exclude figures obtained within 3 days before and 5 days after the onset of menstruation, we find that the greatest deviation from this average takes place within the menstrual period. If, however, we take as a "normal"

TABLE I.

Before Breakfast Blood Sugar Data of Group I.

M., age 24 yrs.			C., age 24 yrs.			F., age 31 yrs.			Remarks.
Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.	
1923		per cent	1923		per cent	1924		per cent	
Sept. 29	-4	0.088	Oct. 12	-5	0.088				Constant Diet I.
Oct. 2	-1	0.092	" 16	-1	0.092				
" 4	+1	0.093	" 17	0	0.102				Mixed.
" 6	+3	0.092	" 19	+2	0.101				
" 10	+7	0.095	" 23	+6	0.100				
" 17	-13	0.081							
" 24	-6	0.085							
			1924						
			Sept. 5	-6	0.109	Sept. 16	+1	0.091	Constant Diet II.
			" 8	-3	0.101	" 18	+3	0.093	
			" 11	0	0.097	" 22	+7	0.095	Purine-low.
			" 12	+1	0.099	" 25	+10	0.094	
			" 13	+2	0.098	" 29	-7	0.097	
			" 15	+4	0.097	Oct. 3	-3	0.091	
			" 18	+7	0.097	" 6	0	0.091	
			" 22	+11	0.099	" 9	+3	0.098	
			" 25	+14	0.099	" 10	+4	0.099	
			" 29	+18	0.098	" 11	+5	0.090	
			Oct. 3	+22	0.094	" 13	+7	0.100	
						" 16	+10	0.096	
						" 18	+12	0.090	
			" 30	-5	0.097	" 21	-11	0.093	
			Nov. 3	-2	0.094	" 25	-7	0.088	
			" 5	0	0.097	" 27	-5	0.098	
			" 6	+1	0.107	" 28	-4	0.092	
			" 7	+2	0.101	" 29	-3	0.089	
			" 8	+3	0.102	" 31	-1	0.097	
			" 10	+5	0.102				
			" 12	+7	0.092				
			1923						
Nov. 1	0	0.100	Nov. 12	0	0.108	Jan. 18	-5	0.087	Constant Diet III.
" 3	+3	0.090	" 13	+1	0.101	" 20	-3	0.100	
" 7	+7	0.090	" 15	+3	0.096	" 23	0	0.094	

TABLE I—*Concluded.*

M., age 24 yrs.			C., age 24 yrs.			F., age 31 yrs.			Remarks.
Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.	
1923		per cent	1923		per cent	1924		per cent	
Nov. 14	+14	0.091	Nov. 17	+5	0.089	Jan. 25	+2	0.093	High protein.
			" 20	+8	0.090	" 26	+3	0.094	
			" 22	+10	0.090	" 28	+5	0.099	
						" 30	+7	0.100	
						Feb. 2	+10	0.095	Purine-low.
						" 4	+12	0.098	
						" 6	+14	0.089	
Nov. 22	-14	0.080	Nov. 26	-14	0.095	" 8	-11	0.090	Constant Diet IV.
" 26	-10	0.092	Dec. 3	-7	0.098	" 13		6 0.092	
Dec. 3	-2	0.090	" 7	-2	0.097	" 18	-1	0.102	
" 5	0	0.092	" 9	0	0.096	" 19	0	0.093	Low protein.
" 6	+1	0.097	" 10	+1	0.101	" 21	+2	0.094	
" 8	+3	0.088	" 12	+3	0.094	" 23	+4	0.105	
" 10	+5	0.094	" 14	+5	0.099	" 27	+7	0.101	Purine-low.
" 12	+7	0.093	" 15	+6	0.100	Mar. 3	+12	0.097	
						" 6	-7	0.089	
						" 8	-5	0.094	
						" 12	0	0.094	
						" 13	+1	0.093	
						" 15	+3	0.089	
						" 17	+5	0.089	
						" 21	+9	0.089	

* The numbers in this column indicate days before (—) and days after (+) the onset of menstruation. Values for bloods taken during menstruation are printed in bold faced type.

level, the average of all determinations for the individual in any given month, we find the average menstrual deviations slightly smaller. This is because we have not only higher, but also, frequently, lower values during menstruation than at any other time. It must be stated, unfortunately, that a set of averages made up in this way can hardly be taken as entirely representative, because

TABLE II.
Before Breakfast Blood Sugar Data of Group II.

Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.
N., age 29 yrs.			Nd., age 31 yrs.			Eb., age 24 yrs.		
1923		per cent	1924		per cent	1923		per cent
Oct. 31	-2	0.091	Feb. 8	-8	0.091	Nov. 15	+2	0.096
Nov. 3	+2	0.091	" 15	-1	0.104	" 17	+4	0.080
" 5	+4	0.091	" 16	0	0.100	" 20	+7	0.089
" 7	+6	0.098	" 18	+2	0.102	Dec. 10	-2	0.093
" 16	+16	0.089	" 20	+4	0.096			
1924			" 27	+11	0.100	Pe., age 20 yrs.		
Mar. 5	-12	0.105	Mar. 12		0.095			
" 13	-4	0.099	Mb., age 22 yrs.			1923		
" 18	+1	0.100				Nov. 20	-13	0.087
" 19	+2	0.102				Dec. 4	+1	0.081
" 21	+4	0.102	1924			" 6	+3	0.092
" 24	+7	0.092	Feb. 11	-10	0.090	" 8	+5	0.085
Ij., age 20 yrs.			" 17	-4	0.102	Pt., age 23 yrs.		
1923			" 21	0	0.092			
Nov. 10	-12	0.104	" 25	+4	0.106	1924		
" 20	-2	0.092	Mar. 3	+11	0.104	Mar. 12	0	0.100
" 22	0	0.087	" 12	-5	0.093	" 19	+7	0.100
" 24	+2	0.093	" 17	0	0.094	" 21	+9	0.100
" 26	+4	0.093	" 19	+2	0.095	" 24	+12	0.102
Dec. 10	+19	0.095	" 21	+4	0.091	X., age 22 yrs.		
			" 24	+7	0.091			
Pv., age 19 yrs.			Pl., age 30 yrs.			1924		
1923			1923			Feb. 18	-3	0.107
Nov. 15	-13	0.093	Oct. 17	-10	0.084	" 21	0	0.095
" 28	0	0.102	" 23	-4	0.094	" 23	+2	0.099
" 30	+2	0.098	" 26	-1	0.093	" 25	+4	0.098
Dec. 2	+4	0.091	" 27	0	0.080	Mar. 3	+11	0.101
" 5	+7	0.089	" 28	+1	0.089	Ib., age 22 yrs.		
Nk., age 21 yrs.			" 29	+2	0.100			
1924			" 30	+3	0.092	1925		
Feb. 7	+1	0.100	" 31	+4	0.085	Mar. 25	-15	0.096
" 9	+3	0.102	Nov. 5	+9	0.093	" 28	-12	0.097
			" 7	+11	0.092			

*The numbers in this column indicate days before (-) and days after (+) the onset of menstruation. Values for bloods taken during menstruation are printed in bold faced type.

TABLE II—*Continued.*

Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.
Nk.— <i>Concluded.</i>			Kp., age 34 yrs.			Ib.— <i>Concluded.</i>		
1924		per cent	1924		per cent	1925		per cent
Feb. 11	+5	0.104	Sept. 1	0	0.098	Apr. 1	—8	0.090
" 13	+7	0.105	" 2	+1	0.103	" 6	—3	0.096
" 20	+14	0.094	" 4	+3	0.098	" 8	—1	0.097
" 27	—8	0.117	" 8	+7	0.084	" 10	+1	0.095
Mar. 3	—3	0.107	" 15	+14	0.084	" 13	+4	0.099
" 6	0	0.096	" 23	—10	0.098	" 16	+7	0.100
" 8	+2	0.107	Oct. 1	+2	0.095	" 20	+11	0.098
						" 25	+16	0.094
Hs., age 27 yrs.			Di., age 21 yrs.			Fe., age 19 yrs.		
1925			1924			1924		
Apr. 2	—8	0.089	Sept. 23	—2	0.098	Sept. 1	—9	0.103
" 9	—1	0.084	" 25	0	0.094	" 6	—4	0.093
" 11	+1	0.091	" 27	+2	0.092	" 10	0	0.087
" 14	+4	0.092	" 29	+4	0.091	" 11	+1	0.102
" 16	+6	0.085	Oct. 3	+8	0.094	" 13	+3	0.100
" 21	+11	0.089	" 8	+13	0.093	" 16	+6	0.095
" 25	+15	0.088	" 17		0.094	" 23	+13	0.106
E. W., age 22 yrs.			Db., age 23 yrs.			Oct. 7	—6	0.098
1924			1924			" 13	0	0.101
Oct. 3	—10	0.079	Sept. 30	—10	0.096	" 16	+3	0.109
" 8	—5	0.092	Oct. 3	—6	0.089	" 21	+8	0.092
" 13	0	0.090	" 10	+1	0.091	N.O., age 36 yrs.		
" 15	+2	0.085	" 13	+4	0.096	1925		
" 18	+5	0.087	" 17	+8	0.090	Mar. 19	—14	0.095
" 22	+9	0.098	" 21	+12	0.091	" 24	—9	0.097
" 27	+14	0.097	" 25	—10	0.090	" 26	—7	0.093
Sp., age 28 yrs.			" 30	—5	0.092	Apr. 2	0	0.089
1924			Nov. 6	+1	0.101	" 9	+7	0.096
Sept. 1	—19	0.096	" 8	+3	0.096	" 16	+14	0.094
" 16	—14	0.099	" 11	+6	0.114			
" 25	—5	0.097						
" 30	0	0.097						

TABLE II—*Concluded.*

Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.	Date.	Date* in relation to menstruation.	Blood sugar.
Sp.— <i>Concluded.</i>			Cs., age 20 yrs.			Cr., age 25 yrs.		
1924		per cent	1925		per cent	1924		per cent
Oct. 2	+2	0.096	Jan. 26		0.098	Nov. 12	−21	0.092
“ 9	+9	0.099	“ 29		0.099	“ 19	−14	0.103
“ 11	+11	0.087	Feb. 3	−17	0.102	“ 26	−7	0.107
“ 23	−5	0.097	“ 6	−14	0.099	Dec. 4	0	0.094
“ 25	−3	0.091	“ 10	−10	0.103	“ 8	+4	0.105
“ 28	0	0.095	“ 13	−7	0.097	Mbe., age 19 yrs.		
“ 29	+1	0.093	“ 17	−3	0.093	1925		
“ 31	+3	0.090	“ 20	0	0.104	Jan. 27	−2	0.091
Nov. 3	+6	0.101	“ 21	+1	0.105	“ 30	+1	0.089
“ 5	+8	0.096	“ 24	+4	0.097	Feb. 3	+5	0.094
“ 11	+13	0.084	“ 27	+7	0.096	“ 6	+8	0.093
1925			Mar. 3	+11	0.095	“ 10	+12	0.099
Jan. 29	−8	0.091	Fs., age 33 yrs.			“ 13	−18	0.096
Feb. 6	0	0.096	1925			“ 17	−14	0.097
“ 10	+4	0.098	Jan. 28	−10	0.103	“ 20	−11	0.090
“ 14	+8	0.099	Feb. 3	−4	0.098	“ 27	−4	0.092
“ 16	+10	0.095	“ 7	0	0.089	Mar. 3	0	0.089
“ 20	+14	0.090	“ 9	+2	0.087	“ 5	+2	0.096
“ 25	−7	0.093	“ 14	+7	0.089			
“ 26	−6	0.090	“ 18	+11	0.093			
Mar. 3	−1	0.098	“ 25	−5	0.088			
“ 4	0	0.099	“ 27	−3	0.091			
“ 6	+2	0.097	Mar. 3	−1	0.097			
“ 9	+5	0.098	“ 6	−4	0.094			

the number of intermenstrual determinations for some of the subjects was not large enough.

The tolerance tests (see Table IV and Figs. 1 to 3), on the other hand, seem to give more conclusive evidence of cyclic variation. Inspection of the tables will show that the lowest peak of the blood sugar curves following the ingestion of 1.75 gm. of glucose per kilo of body weight came during or immediately

before the menstrual period. The highest blood sugar values obtained for each individual were in the tests made just before or just after this time. This would seem to indicate that the mechanism by which glucose is removed from the blood stream is, in some way, altered at the time of menstruation.

TABLE III.

Summary of Results of before Breakfast Blood Sugar Determinations in Normal Individuals.*

Total No. of determinations included	315
No. of menstrual periods included	49
“ “ subjects studied	26
Average fasting blood sugar value	0.0949 per cent.
“ intermenstrual value	0.0941 “ “
“ menstrual value	0.0958 “ “
“ of highest menstrual values for each period observed	0.099 “ “
Average of highest intermenstrual values for each period observed	0.098 “ “
Average of lowest menstrual values for each period observed	0.092 “ “
Average of lowest intermenstrual values for each period observed	0.090 “ “
Average of greatest menstrual deviations from inter- menstrual average	0.0067 “ “
Average of greatest intermenstrual deviations from intermenstrual average	0.0049 “ “
Average of greatest menstrual deviations from general average	0.0056 “ “
Average of greatest intermenstrual deviations from general average	0.0060 “ “

* The results of determinations made on bloods from Group III are omitted in computing these averages because there is some reason to believe that the individuals composing this group were not to be considered as normal.

Perhaps the most interesting and significant thing to be observed from these curves, is, however, the tendency to extremely low blood sugar values in the period from 1 to 2 hours after the ingestion of the test amount of glucose, during menstruation. Subject N.O. gave blood sugar values at this time which approach the lower limits of the range which is considered clinically safe after the giving of insulin. This subject had, however, no abnormally low

	Oct. 16	+4	0.106	0.122	0.090	0.085	0.097		97	98	97	100	97	25.9	9.8	18.6
	" 21	+9	0.092	0.115	0.079	0.077	0.081		96	96	98	94	98	15.3	12.5	16.6
	" 28	+16	0.093	0.103	0.075	0.084	0.085		96	99	99	99	98	10.1	13.8	11.8
	<i>1925</i>															
N.O.	Mar. 19	-14	0.095	0.116	0.067	0.063	0.078		100	98	99	99	97	14.4	15.5	14.1
36 yrs.	" 26	-7	0.093	0.151	0.064	0.057	0.075		100	98	100	100	98	11.5	10.6	15.3
	Apr. 2	0	0.089	0.102	0.067	0.058	0.068		96	95	93	91	93	11.7	14.0	10.3
	" 9	+7	0.096	0.141	0.093	0.088	0.068		96	92	95	93	92	7.9	18.0	10.9
	" 16	+14	0.094	0.112	0.079	0.070	0.074		93	97	91	91	89	9.9	17.6	11.7
	<i>1924</i>															
Cp.	Nov. 12	-7	0.092	0.168	0.153	0.083	0.076		105	98	100	101	102	21.2	44.5	12.9
25 yrs.	" 19	-14	0.103	0.128	0.159	0.106	0.083		110	111	108	108	106	23.2	43.7	17.8
	" 26	-7	0.107	0.128*	0.128*	0.148	0.078		108	107	107	105	109	19.0	31.7	32.9
	Dec. 4	0	0.094	0.120	0.105	0.082	0.085		102	100	98	97	97	10.1	26.6	21.2
	" 8	+4	0.105	0.120*	0.120*	0.099	0.098		99	97	97	95	95	30.8	52.5	23.4
	<i>1925</i>															
Fs.	Nov. 11	-17	0.098	0.132	0.116	0.085	0.099		95	97	95	95	95	16.5	14.9	16.4
32 yrs.	" 20	-8	0.097	0.123	0.095	0.088	0.076		97	93	96	95	95	10.7	13.6	17.9
	" 25	-3	0.107	0.142	0.093	0.088	0.086		94	90	95	92	94	16.6	37.9	23.2
	" 28	0	0.100	0.123	0.126*	0.126*	0.077		94	100	93	93	94	16.2	18.6	19.7
	Dec. 2	+4	0.094	0.120	0.074	0.083	0.096		92	90	95	92	90			
	<i>1925</i>															
	Jan. 28	-10	0.103	0.112	0.110	0.085	0.076							24.9	16.4	18.1
	Feb. 7	0	0.089	0.105	0.075	0.074	0.065		89	93	94	91	89	8.3	41.7	21.5
	" 9	+2	0.087	0.124	0.089	0.075	0.098		93	97	94	92	94	21.6	21.0	19.7
	" 18	+11	0.093	0.119	0.099	0.097	0.075		92	89	90	92	89	38.2	40.2	23.0
	" 25	+18	0.088	0.110	0.114	0.074	0.089							45.4	16.8	21.7

TABLE IV—*Concluded.*

Subject.	Date.	Blood sugar.						Hemoglobin.				Urine sugars.					
		0		½ hr.	1 hr.	1½ hrs.	2 hrs.	2½ hrs.	0		½ hr.	1 hr.	1½ hrs.	2 hrs.	0	1 hr.	2 hrs.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mg.	mg.	mg.
Hs. 27 yrs.	1925																
	Apr. 2	-8	0.089	0.110	0.088	0.083	0.078		101	99	100	104	102	11.8	13.9	11.1	
	" 9	-1	0.084	0.109	0.060	0.071	0.073		102	105	106	103	101	7.3	13.2	9.7	
	" 11	+1	0.091	0.115	0.101	0.078	0.088		97	101	99	100	99	9.7	16.9	15.1	
	" 14	+4	0.092	0.128	0.116	0.078	0.100		98	101	97	100	97	12.6	12.4	11.3	
	" 21	+11	0.089	0.114	0.088	0.078	0.089		98	99	102	99	96	9.5	19.5	13.2	
Ib. 22 yrs.	Mar. 25	-14	0.096	0.114	0.117	0.122	0.109	0.099	103	100	98	100		43.4	42.1	18.4	
	Apr. 1	-8	0.090	0.105	0.115	0.100	0.096	0.076	96	94	93	97	99	15.3	30.4	19.9	
	" 8	-1	0.097	0.100	0.122	0.100	0.089		103	100	98	101	98	12.2	17.9		
	" 10	+1	0.095	0.169	0.136	0.126	0.096		101	98	101	100	102	10.7	22.1	14.4	
	" 13	+4	0.099	0.107	0.113	0.118	0.111		98	101	97	101	96	9.7	37.5	30.8	
	" 20	+11	0.098	0.123	0.174	0.157	0.118		101	109	103	104	104	6.9	3.6	3.3	

* These figures represent one analysis made on the blood sample taken 45 minutes after the one given just before.

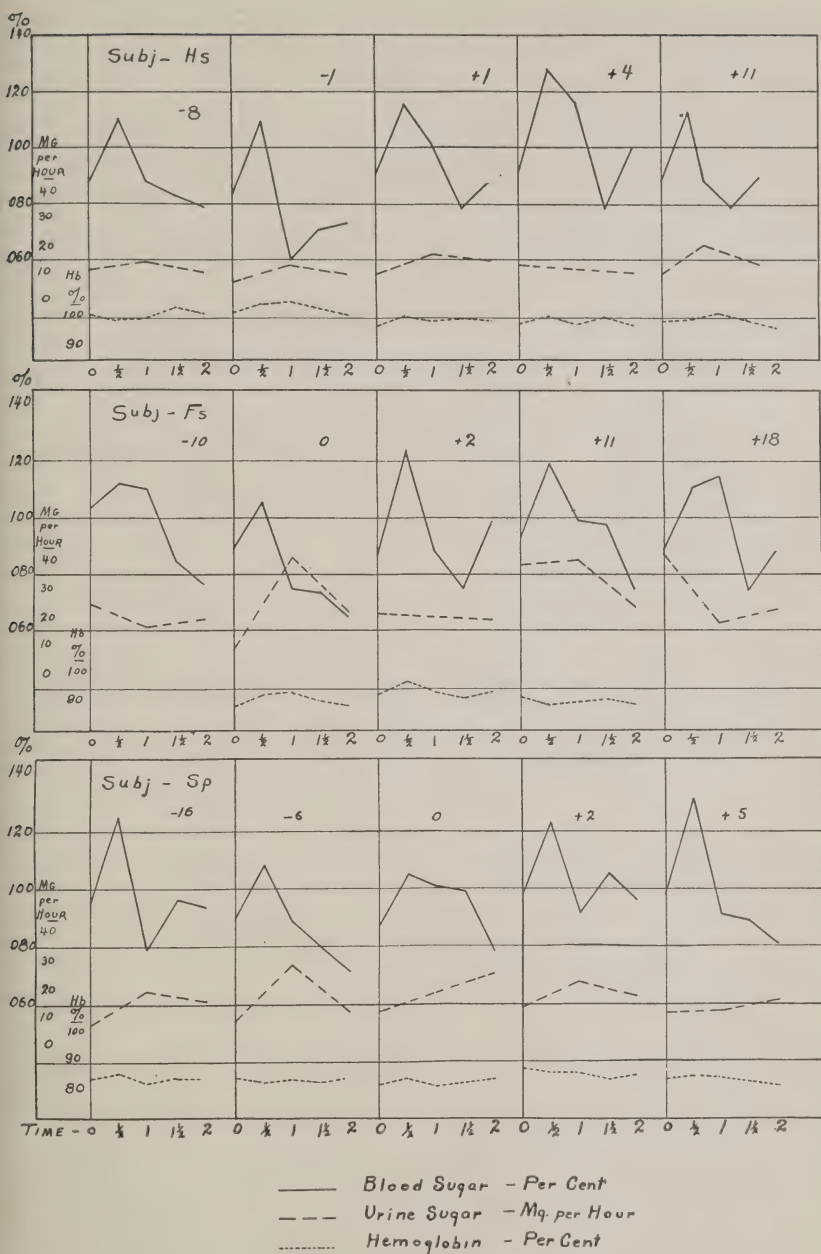


FIG. 1. Tolerance curves. Ingestion of 1.75 gm. of glucose per kilo of body weight.

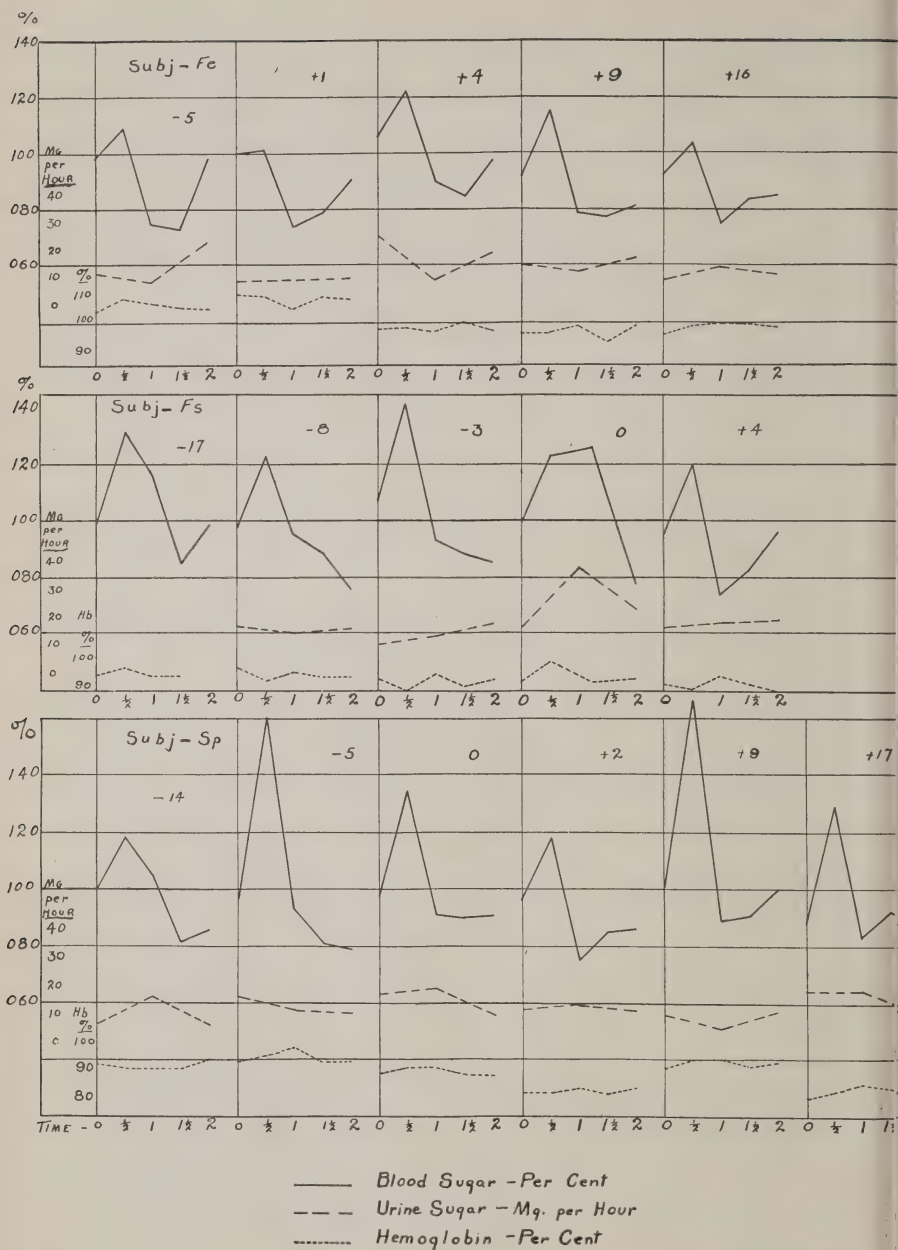


FIG. 2. Tolerance curves. Ingestion of 1.75 gm. of glucose per kilo of body weight.

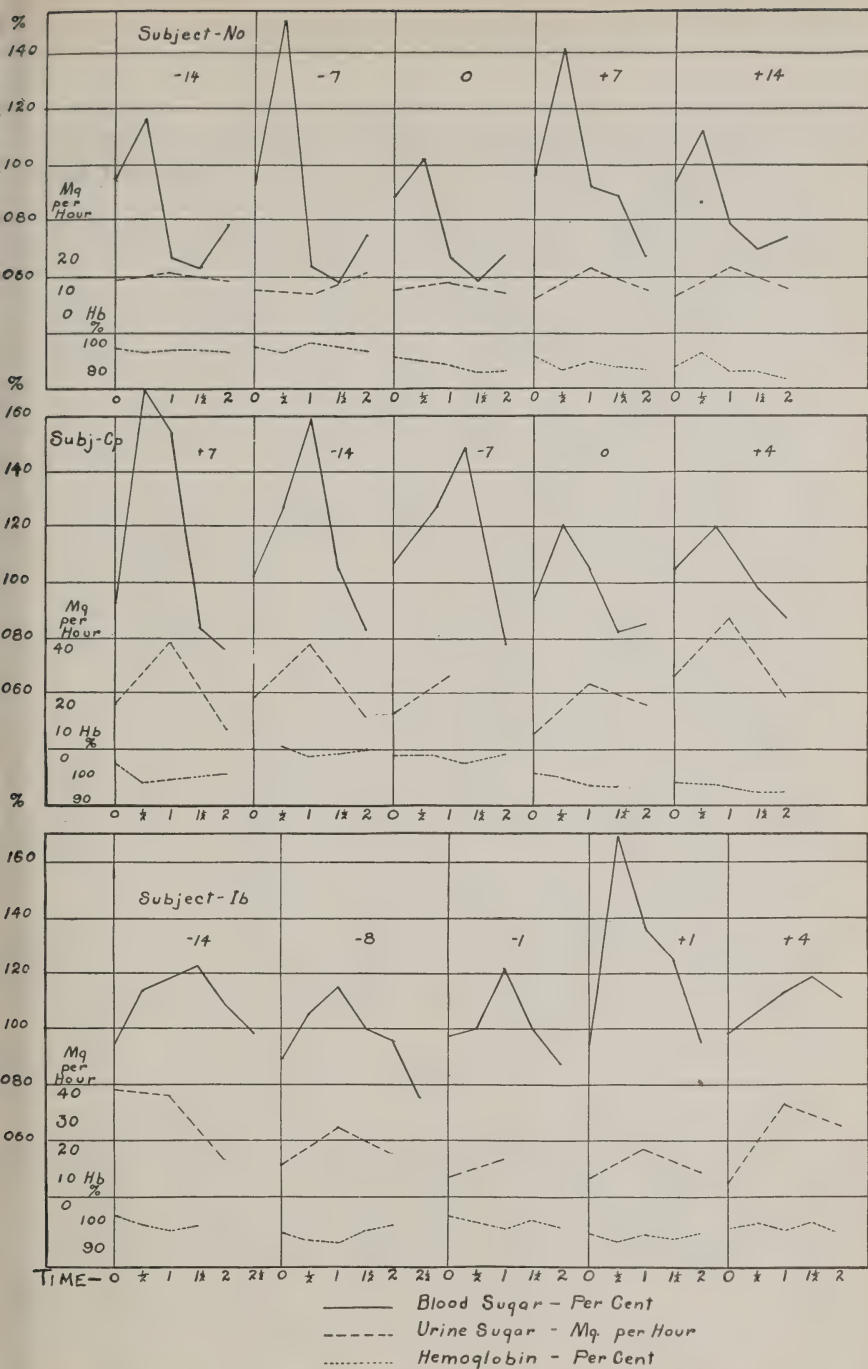


FIG. 3. Tolerance curves. Ingestion of 1.75 gm. of glucose per kilo of body weight.

values for fasting blood sugar, and gave no history of physical abnormality. She did, however, complain of extreme hunger during the latter part of each of these test periods. Subject Fs., who also responded to the tolerance test with very low blood sugar values, stated that she was always ravenously hungry during her menstrual periods. Questioning of a number of other subjects has, moreover, led to the conclusion that, in cases where menstruation was not accompanied by nausea or cramps, this tendency to unusual hunger 2 or 3 hours after a meal was often observed during the period. The hunger may possibly be associated with increased tonicity of the smooth musculature as a whole, and, according to Sevringhaus (22) considered as an accompanying factor rather than a result of the hypoglycemia. There is, on the other hand, a possibility that this delayed relief from hypoglycemia during the menstrual period, with the unusually low blood sugar values following the ingestion of the test carbohydrate, may indicate that the pancreatic response is in some way altered at this time. Again, the fact that the lowest minima were obtained for the oldest subjects (N.O., aged 36 years; and Fs., aged 32 years) may possibly have some significance.

The types of curves obtained just before and just after the menstrual periods hardly correspond to those accepted as typical of hyperthyroidism, in that the return to the fasting level of blood sugar is too rapid. During menstruation, moreover, there is a tendency to develop lower minima as well as maxima.

The writers feel inclined to think that the seemingly contradictory results of the previous investigators quoted may, perhaps, be explained by the fact that no one of them has ever made a sufficiently large series of determinations on any one subject. There is, however, a possibility that the reaction of the normal individual to sugar ingestion, if it does involve added production of insulin or the need for added insulin during the menstrual period, may be radically different from the response of the person whose capacity for producing insulin is curtailed by disease. It is hoped that it may be possible, at a future time, to study the effect of menstruation on the threshold of ketosis.

SUMMARY AND CONCLUSIONS.

On the basis of a total of more than 300 determinations of "before breakfast" blood sugar, covering 49 menstrual periods in 26 normal women there seems to be no ground for conclusion that there is a consistent cyclic variation in the fasting blood sugar level in women. The average values observed during menstruation are slightly higher than those for the intermenstrual period. However, there are a greater number, not only of high values, but also of low values, during the menstrual period than at any other time. Hence it is believed that, in making single determinations of blood sugar for purposes of clinical diagnosis, the time of the menstrual period should, in as far as possible, be avoided. This conclusion is borne out by a limited number (approximately 50) of additional observations on subjects not to be rated as strictly normal physically.

The ingestion of 1.75 gm. of glucose per kilo of body weight has, however, led in the ten cases observed, not only to smaller initial increases in blood sugar, but also to a greater degree of secondary hypoglycemia during the menstrual period than at any other time of the month. The effect noted immediately before and immediately after this time is just the opposite, *i.e.* a lessened tolerance, while the ingestion of glucose in the intermenstrual period has an intermediate effect. This smaller increase and greater decrease in blood sugar following glucose ingestion during menstruation suggests an altered functioning of the pancreas, coincident with the time of menstruation. Speculation as to whether or not this is the result of an altered ovarian or suprarenal secretion seems hardly justified by the evidence at hand.

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THE METABOLISM OF SULFUR.

IX. THE EFFECT OF REPEATED ADMINISTRATION OF SMALL AMOUNTS OF CYSTINE.*

BY HOWARD B. LEWIS.

WITH THE COOPERATION OF HELEN UPDEGRAFF.

(From the Laboratory of Physiological Chemistry, University of Michigan, Ann Arbor.)

(Received for publication, June 18, 1925.)

In earlier experiments in which large single doses of cystine (4 to 5 gm.) were fed to rabbits, it was observed that the animals usually developed an albuminuria and died within a short time. In later experiments, in which the influence of the amino acid content of the protein of the diet on the composition of the tissues was under investigation, similar results were noted after ingestion of cystine. The observations have led to a study of the changes in the blood and urine following the feeding of cystine in small doses (usually 0.5 to 1.0 gm. per kilo of body weight) which is here reported.

The experimental animals were rabbits which were either fasted or maintained on constant diets of oats and cabbage throughout the experimental periods. The urine was collected in 24 hour periods as usual. Cystine was dissolved in the calculated amount of a standard solution of sodium hydroxide, this solution was diluted to about 50 cc., and administered through a stomach tube. Total nitrogen was determined by the Kjeldahl method; creatinine and amino acid nitrogen, by the methods of Folin; and cystine, by the method of Looney. For the analysis of the blood, the usual procedure of Folin and Wu was followed. The sulfur partition was determined by the usual gravi-

* The substance of this paper was presented before the American Society of Biological Chemists at Washington, December 29-31, 1924 (Lewis, H. B., *J. Biol. Chem.*, 1925, lxiii, p. xx).

metric methods, except in one experiment (Rabbit F, Table III), in which the volumetric procedure of Fiske was followed. For the determination of total sulfur Miss Denis' modification of Benedict's copper nitrate fusion method was used. Unoxidized ("neutral") sulfur was determined by difference.

In order to rule out the so called "spontaneous" nephritis which may occur in laboratory animals, the urine in each case was carefully examined for protein and casts, prior to the beginning of each experiment. In addition, the normal days of each series furnished additional controls, as evidence that the effects observed after cystine feeding were the results of the experimental procedure and not of preexisting conditions of the animals. For the detection of protein in the urine, heat coagulation, Heller's ring test, and the picric acid test were employed. At the termination of each experiment, the kidneys were studied pathologically in the laboratory of the Department of Pathology. We are indebted to Dr. A. S. Warthin for the pathological reports on these tissues.

The results of the experiments are shown in Tables I to IV. These represent typical experiments, similar results having been obtained with other animals. In Tables I and II, cystine was fed to fasting rabbits. Rabbit D (Table I) received 2.0, 3.0, and 4.0 gm. of cystine *per os* on 3 successive days. The animal died about 16 hours after the last administration of cystine. Protein and casts appeared in the urine of the 1st feeding day. Despite the fact that on the 2nd feeding day about 350 mg. of nitrogen were fed, the nitrogen elimination decreased to 0.729 gm. The simultaneous decrease in the creatinine elimination also points to a failure of the kidney function. Despite the large doses of the amino acid fed, the amino acid nitrogen of the urine showed little increase and this slightly increased excretion could not be accounted for by the cystine eliminated by the kidneys.

Similar results were obtained with Rabbit E (Table II), also a fasting animal. The animal died 12 hours after the feeding of the third successive dose of cystine. As before, protein and casts were observed in the urine of the 1st day of cystine feeding and continued throughout the experiment. The same striking failure of elimination by the kidneys is seen on the 2nd and 3rd

days in which the total nitrogen excretions dropped to 0.368 and 0.161 gm. (12 hour specimen) respectively, while the creatinine eliminations also decreased (0.075 and 0.022 gm.). The excretions of amino acid nitrogen and cystine were not markedly changed. On the morning of the day following the second ad-

TABLE I.
Rabbit D.

Date.	Weight.	Nitrogen.	Creatinine.	Amino nitrogen.	Cystine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Remarks.
1924	kg.	gm.	gm.	mg.	mg.	gm.	gm.	gm.	
Mar. 16-17	2.05	1.622	0.117	8.2	13.4	0.125	0.101	0.024	Fasting.
" 17-18	2.00	1.087	0.084	4.4	5.5	0.061	0.052	0.009	"
" 18-19	1.95	1.248	0.085	12.3	17.5	0.456	0.369	0.087	2.0 gm. cystine.
" 19-20	1.85	0.729	0.049	18.4	18.0	0.512	0.341	0.171	3.0 " "
" 20-21*	1.75	0.690	0.030	29.3	8.9	0.455	0.226	0.229	4.0 " "

* Animal died during night probably about 12.30 a.m. Urine specimen not 24 hours.

TABLE II.
Rabbit E.

Date.	Weight.	Nitrogen.	Creatinine.	Amino nitrogen.	Cystine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Remarks.
1924	kg.	gm.	gm.	mg.	mg.	gm.	gm.	gm.	
May 1-2	3.00	0.698	0.093	6.4	11.6	0.077	0.057	0.020	Fasting.
" 2-3*	2.90	1.046	0.147	11.63	11.3	0.075	0.063	0.012	"
" 3-4	2.80	1.269	0.133	6.62	9.8	0.086	0.075	0.011	"
" 4-5	2.70	1.004	0.111	8.13	12.7	0.409	0.350	0.059	2.0 gm. cystine.
" 5-6	2.65	0.368	0.075	5.94	12.9	0.379	0.304	0.075	3.0 " "
" 6-7†	2.60	0.161‡	0.022	11.63		0.094	0.081	0.013	2.8 " "

* Blood, May 2, 9.00 a.m., non-protein nitrogen, 42.11 mg.; amino nitrogen, 8.59 mg.

† Blood, May 6, 9.00 a.m., before cystine feeding, non-protein nitrogen, 156.3 mg.; amino nitrogen, 191.14 mg.

‡ Animal died 8.55 p.m., May 6. Blood from heart at point of death, non-protein nitrogen, 250.0 mg.; amino nitrogen, 58.10 mg. Urine, a 12 hour sample.

ministration of cystine and 24 hours after this second administration, the non-protein nitrogen of the blood had increased to almost four times the normal value. The rise in blood amino nitrogen was not so marked.

TABLE III.

Rabbit F.

Date.	Weight.	Nitrogen.	Creatinine.	Amino nitrogen.	Cystine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Remarks.
1924	kg.	gm.	gm.	mg.	mg.	gm.	gm.	gm.	
May 22-23	1.30	0.757	0.071	6.36	6.5	0.116	0.104	0.012	45 gm. cabbage and oats.
" 23-24	1.30	0.876	0.059	9.82	9.0	0.097	0.079	0.018	45 gm. cabbage and oats.
" 24-25	1.30	0.587	0.066	9.29	8.9	0.080	0.080	0.020	45 gm. cabbage and oats.
" 25-26*	1.30	0.685	0.062	6.76	6.0	0.088	0.075	0.013	45 gm. cabbage and oats.
" 26-27	1.30	0.708	0.049	10.95	5.7	0.300	0.198	0.102	1.0 gm. cystine. Ate food.
" 27-28	1.25	0.306	0.040	8.00	7.1	0.249	0.168	0.081	1.0 gm. cystine. Refused food.
" 28-29†	1.20	0.290	0.031	5.22	6.8	0.219	0.147	0.072	1.5 gm. cystine. Refused food.
" 29-30	1.15	0.586	0.041	7.50	6.4	0.209	0.180	0.029	No cystine. Fasting.
" 30-31	1.10	0.790	0.046	7.00	7.2	0.139	0.102	0.037	No cystine. Fasting.
June 1	Dead in cage.								

* Blood, May 26, 9.00 a.m., prior to cystine administration, non-protein nitrogen, 42.12 mg.; amino nitrogen, 14.3 mg.

† Blood, May 28, 9.00 a.m., prior to cystine, non-protein nitrogen, 104.47 mg.; amino nitrogen, 14.0 mg.

Similar results are shown in Tables III and IV in which the animals to which cystine was fed received a diet of oats and cabbage. These animals also showed protein and casts in the urine. The urine and blood pictures are similar to those of the fasting rabbits, decreased elimination of nitrogen and creatinine by the kidneys, and a rise in the non-protein nitrogen of

the blood. Rabbit F (Table III) lived for 3 days after the last feeding of cystine. After the administration of the cystine was discontinued, the elimination of nitrogen gradually increased, until it returned to the normal value. The excretion of creatinine, although slightly increased, failed to return to normal, prior to the death of the animal. No marked changes in amino acid nitrogen or cystine excretions were observed.

These and other similar experiments all agree in demonstrating that oral administration of cystine in amounts usually considered non-toxic resulted in serious injury to the kidneys as evidenced

TABLE IV.

Rabbit G.

Date.	Weight.	Nitrogen.	Creatinine.	Amino nitrogen.	Remarks.
1924	kg.	gm.	gm.	mg.	
June 6-7	2.30	1.000	0.073	10.31	50 gm. cabbage and oats.
" 7-8	2.25	0.920	0.083	9.7	50 " " " "
" 8-9*		0.972	0.089	8.89	50 " " " "
" 9-10	2.10	0.920	0.088	13.10	1.5 gm. cystine. Ate food.
" 10-11†	2.05	1.003	0.087	16.95	2.0 " " " cabbage, no oats.
" 11-12	1.95	0.761	0.081	15.15	2.5 gm. cystine. Ate cabbage, slight amount of oats.
" 12-13‡		0.152	0.026	7.37	3.0 gm. cystine. Fasting.

* Blood, June 9, 9.00 a.m., prior to cystine, non-protein nitrogen, 49.0 mg.; amino nitrogen, 4.00 mg.

† Blood, June 11, 9.00 a.m., prior to cystine, non-protein nitrogen, 75.8 mg.; amino nitrogen, 12.15 mg.

‡ Died during night. Dead in cage in morning. In good condition at 8.45 p. m. Urine in bladder 50 cc.

by both urinary and blood pictures. Further confirmation of this is afforded by the results of the pathological examination of the kidneys (details in the appendix to this paper), which revealed injury to the kidneys in every case. It might be considered that the effect was not specific for cystine, but was due to the administration of large amounts of amino acids with the resultant overwhelming of the organism by the amino acids themselves or their nitrogenous products of catabolism. We do not consider, however, that this explanation is correct, since we have repeatedly administered greater amounts of nitrogen as

glycine or alanine to rabbits than were fed as cystine in the present series, and have not observed the same urinary picture. One such control experiment is recorded in Table V in which glycine, equivalent in its nitrogen content to 2.69 gm. of cystine, was fed for 4 successive days. No protein or casts were present in the urine at any time. In striking contrast to the experiments previously detailed, the extra nitrogen fed was almost quantitatively excreted on the day of feeding and no significant

TABLE V.

Rabbit J.

Date.	Weight.	Total nitrogen.	Creatinine.	Amino nitrogen.	Remarks.
1924	kg.	gm.	gm.	mg.	
Oct. 5-6	2.75	1.173	0.150	12.7	
" 6-7	2.65	1.026	0.143	8.9	
" 7-8	2.60	1.035	0.146	8.3	
" 8-9	2.60	1.100	0.140	8.2	
" 9-10*	2.55	1.290	0.174	23.4	1.68 gm. glycine† <i>per os</i> .
" 10-11	2.55	1.460	0.136	13.4	1.68 " " "
" 11-12	2.55	1.376	0.134	13.0	1.68 " " "
" 12-13	2.55	1.323	0.123	11.0	1.68 " " "
" 13-14‡	2.55	1.125	0.132	7.1	
" 14-15	2.50	1.019	0.130	6.6	

* Blood, Oct. 9, prior to glycine feeding, non-protein nitrogen, 46.8 mg.; amino nitrogen, 11.6 mg.

† In content of nitrogen, 1.68 gm. of glycine are equivalent to 2.69 gm. of cystine.

‡ Blood, Oct. 13, 11.00 a.m., non-protein nitrogen, 51.0 mg.; amino nitrogen, 8.0 mg.

alteration of the creatinine elimination was evident. The blood content in non-protein nitrogen remained normal at the end of the experiment.

Some evidence of the nephrotoxic action of cystine is recorded in the literature. After the intravenous injection of approximately 1.0 gm. of cystine per kilo to a dog¹ death resulted within a short time and autopsy revealed severe hemorrhagic nephritis. Smaller doses produced no similar effects in dogs. A single dose of 2.0 gm. of cystine fed to a rabbit of 2.8 kilos apparently

¹ Blum (1), p. 4.

produced no injury² although examination of the kidneys was not made. Newburgh and Marsh (2) have injected a large number of amino acids intravenously into dogs and rabbits and have studied the resulting changes in the kidney. Of the nephrotoxic amino acids, the action of cystine was very pronounced. On the other hand, Wohlgemuth (3) did not observe any toxic effects after the feeding of much larger amounts of cystine to rabbits than those fed in the present series. The low percentage of the sulfur fed as cystine, which was recovered in the urine after feeding, suggests, however, a possible impairment of kidney function with a delayed excretion of sulfur. The excretion of other products of metabolism was not studied.

One aspect of the excretion of the various forms of sulfur in the present study is worthy of brief comment. It is usually stated that moderate doses of cystine are oxidized almost completely to sulfates by the organism of the rabbit. In the experiments recorded here, the increases in the "neutral" sulfur of the urine following cystine feeding were marked, amounting in some experiments to 20 per cent or more of the ingested sulfur (Tables I and III and other unrecorded experiments). It has usually been assumed that this increase in "neutral" sulfur excretion is due to the presence of unchanged cystine. However, the increase in unoxidized sulfur after cystine feeding is not to be accounted for in this way, since neither the excretion of amino acid nitrogen nor of cystine is significantly altered.

The presence of thiosulfates in the urine in considerable amounts has been observed after feeding cystine to rabbits (3). It seemed possible that the increased "neutral" sulfur fraction of the urine might be due to the presence of these. However, we were unable to demonstrate the presence of thiosulfates in significant amounts in our experiments. If thiosulfates were present, they must have occurred in very small amounts and can hardly account for a significant part of the sulfur fraction under discussion. One other possible source of the unoxidized sulfur must also be considered. The urines, after the ingestion of cystine, contained protein, a sulfur-containing organic complex. This protein was removed before analysis as completely as possible by heat coagulation followed by treatment with alumina cream. It is possible that small

² Blum (1), p. 8.

amounts of protein were not removed by this treatment and that the extra "neutral" sulfur was protein sulfur, which had its origin in some non-coagulable protein fraction of the urine. In view of the low sulfur content of the ordinary proteins, it would be necessary to assume the presence of a very considerable amount of such a protein in order to account for 100 mg. or more of the extra "neutral" sulfur, an amount of protein which would hardly escape detection. Moreover, in experiments in which the animals survived several days following the last ingestion of cystine, although protein was still present in the urine, the neutral sulfur dropped to almost the normal figure, after the administration of cystine was discontinued. Such an experiment is recorded in Table III. In another experiment, the details of which are not presented, the "neutral" sulfur of the fore period averaged 0.020 gm. daily; increased to 0.107, 0.162, 0.073, and 0.131 gm. after feeding of 2.1, 2.1, 1.4, and 2.1 gm. of cystine; and returned to 0.043, 0.025, and 0.023 gm. after the administration of cystine was stopped. Marked albuminuria was present during the days of cystine feeding and the subsequent days.

Our studies on rabbits are similar to those of Abderhalden and Samuely on dogs (4). After feeding cystine a considerable rise in the "neutral" sulfur fraction of the urine was observed. Thio-sulfates could not be detected nor could cystine be isolated either as the amino acid itself, the benzoyl chloride derivative, or the β -naphthalene sulfonyl chloride derivative. We believe that our experiments in which cystine and amino acid nitrogen were determined quantitatively in the urine and the above mentioned studies of Abderhalden suggest that after cystine feeding, there may be present in the urine a sulfur-containing substance, intermediary between cystine and inorganic sulfates.

SUMMARY.

Cystine has been administered orally as the sodium salt in moderate doses (0.5 to 1.0 gm. per kilo) for successive days to fasting rabbits and to rabbits on a diet of oats and cabbage. Protein and casts appeared in the urine, the excretions of non-protein nitrogen and creatinine were depressed, but no marked loss in cystine as evidenced by determination of the cystine and amino

acid nitrogen content of the urine was observed. The blood showed a marked rise in the non-protein nitrogen. The "neutral" sulfur fraction of the urine was increased, following cystine feeding, but the rise was not due to the presence of cystine. The possible significance of this increase is discussed.

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Pathological Examinations.

Rabbit D.—Kidney shows acute passive congestion. Cloudy swelling of the convoluted tubules with necrosis of the collecting tubules of the medullary rays of the cortex. Collecting tubules filled with casts and desquamated necrosing epithelium, acute parenchymatous degeneration.

Rabbit E.—Kidney shows acute congestion, cloudy swelling, numerous casts not as marked as in Rabbit D. In addition numerous small inflammatory foci indicating that this rabbit had a pyogenic infection.

Rabbit F.—Kidney shows moderate congestion, slight cloudy swelling, great numbers of hyaline casts in both cortex and medulla.

Rabbit G.—Kidneys show localized areas of chronic infection. Several areas of healed focal nephritis in the cortex. Marked congestion, numerous casts. Cloudy swelling. No abscesses.

THE LOSS OF BASES IN DIURESIS AND ITS EFFECT UPON THE ALKALI RESERVE OF THE BLOOD.*

By BYRON M. HENDRIX AND DEA B. CALVIN.

(From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston.)

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In confirmation of Cushny's (1) and Rüdel's (2) suggestions, Hendrix and Sanders (3) have shown that injection of dibasic phosphates caused a marked rise in the titratable acidity and ammonia of the urine, as well as a rise in the alkali reserve of the blood. The same effect was demonstrated when sodium hippurate was injected. In fact, the rise in total acidity (titratable acid plus ammonia) was very nearly equivalent to the total phosphate or hippurate injected. These experiments point to a definite retention of sodium ions, both from the disodium phosphate and from the sodium hippurate. This interchange must be assumed to occur in the tubules, yielding either a hydroxide or bicarbonate. Hendrix and Bodansky (4) have shown that in early experimental uranium nephritis, the base excreted increases markedly at the time when the fall in alkali reserve is most pronounced; that is, during the first 24 hours. In this case, it would seem that the loss of base through the kidney as dibasic phosphate and the salts of organic acids must be held responsible for a large portion of the observed fall in alkali reserve.

In the present work, the diuresis, due to substances which could not yield base to the body, has been studied. In view of the work of Cushny (1) and Rüdel (2), it seemed that diuresis produced by salts of strong acids, and by urea, might give effects rather opposite, with respect to the reaction of the urine, to those observed by Hendrix and Sanders (3) in the case of disodium phosphate and

* This paper has been abstracted from a thesis presented by Mr. Calvin to the Graduate School of the University of Texas as partial fulfillment of the requirements for the degree of Master of Arts.

sodium hippurate. Since the base is absorbed from the tubules, and since the degree of absorption, both of water and of threshold substances, depends upon the rate at which the glomerular fluid passes through the tubules, as well as upon the osmotic concentration of the tubular fluid, it seemed possible that a loss of base should occur in diuresis. The fall in alkali reserve should be transient, and the kidney should not be injured. It is believed that in the experimental work presented here, a fall in alkali reserve due solely to diuresis has been demonstrated, the results depending on a lowered capacity of the tubules to reabsorb the basic ions, as a result of increased flow of glomerular filtrate through the tubules.

EXPERIMENTAL.

Female dogs were used in all the experiments in order that catheterization could be accomplished with ease. They were kept in large metabolism cages for 24 hours previous to the beginning of the experiment, without food, but allowed to have plenty of distilled water. The urine was collected under toluene to prevent putrefaction, and the dogs were catheterized immediately before injection and at the end of each experimental period. Blood was drawn for analysis from the jugular vein at the same time.

In all, four different diuretics were used; namely, 10 per cent sodium nitrate, 8 per cent sodium chloride, 13 per cent sodium sulfate, and 12 per cent urea. These concentrations were approximately isotonic with each other. The amount of solution injected was varied according to the weight of the dog being used, about 150 cc. per 10 kilos of body weight being the usual dose. The solution was always sterilized by boiling, cooled down to body temperature, and injected into the jugular vein. The dogs were not anesthetized at any time for injection. As near as possible to 4 hours after the injection, blood and urine samples were taken for analysis. This was repeated after a 24 hour period had elapsed from the time of injection.

The ammonia was determined by the method of Folin (5), organic acids according to Van Slyke and Palmer (6), the phosphates by titration with uranium acetate, the urinary pH according to Clark's (7) method, and the titratable acidity by a method to be discussed later. The CO₂-combining power of the whole blood was determined by the method of Van Slyke (8), the oxygen-combining power according to Van Slyke (9), and the pH of the plasma by the method of Cullen (10).

In titrating the acidity of the urine, the effect of ammonium salts was taken into account. The following experiment was performed to determine this effect.

Test-tubes of the large size (20×200 mm.) were used. Into each tube were placed 25 cc. of 0.1 M disodium phosphate and 6 drops of 1 per cent phenolphthalein. Six such tubes, constituting the first series, were prepared. To the first tube, no acid was added; to the second, 4 cc.; to the third, 8 cc.; to the fourth, 12 cc.; to the fifth, 16 cc.; and to the sixth, 20 cc. of 0.1 N hydrochloric acid were added. The contents of each tube were titrated with 0.1 N sodium hydroxide to the color produced by 6 drops of 1 per cent phenolphthalein added to a control tube containing 25 cc. of 0.1 M disodium phosphate. In Series 1, the acid added was equivalent to the alkali required to titrate back to the definite phosphate end-point. Five

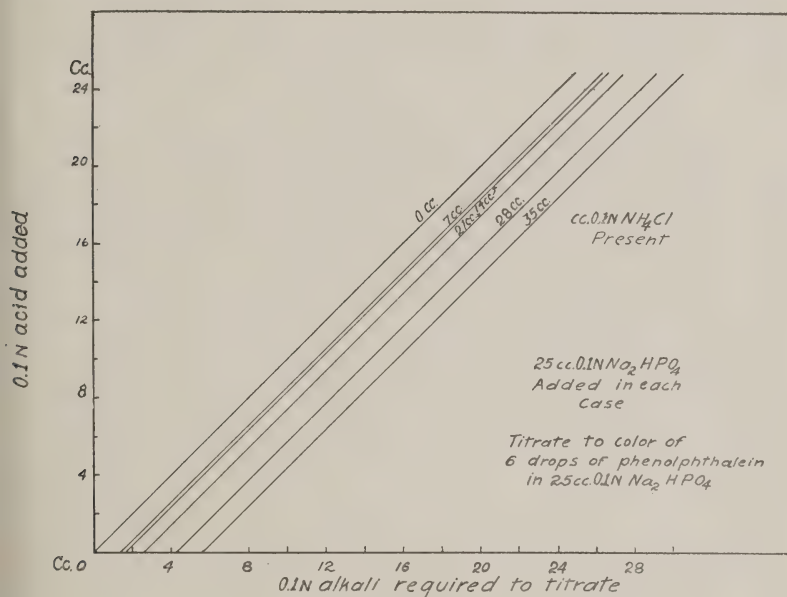


FIG. 1.

other series of six tubes each were prepared, as before, and to Series 2, 0.7 cc.; to Series 3, 1.4 cc.; to Series 4, 2.1 cc.; to Series 5, 2.8 cc.; and to Series 6, 3.5 cc. of M ammonium chloride were added. The contents of each tube of each series were then titrated to the disodium phosphate end-point with 0.1 N sodium hydroxide. A molecular, instead of a 0.1 M solution of ammonium chloride was used in order to keep the volume as nearly constant as possible. The results are shown graphically in Fig. 1.

The results of this experiment were used in all of the experimental work on this problem. The method of urine titration may be outlined in the following way.

25 cc. of urine were pipetted into a 250 cc. Erlenmeyer flask, and 25 cc. of distilled water, 5 cc. of a saturated solution of neutral sodium oxalate, and 6 drops of 1 per cent phenolphthalein were added. The mixture was then titrated with decinormal sodium hydroxide to the color produced in the phenolphthalein-phosphate standard, in order that all the phosphates might be changed into the dibasic form. Since it was desired to calculate the base lost from the difference between titratable acid plus ammonia and phosphates plus organic acids, it was necessary to titrate to an end-point at which the acid phosphates were completely changed into the dibasic form. The ammonia was determined by the aeration method and from this value, the amount of 0.1 N ammonia in 25 cc. of urine was calculated. Knowing this value, and the titration figure on 25 cc. of urine, reference to Fig. 1 gives the actual amount of acid present in the aliquot portion of the urine.

This method proved of much help in obtaining correct values for the content of acid phosphate and eliminated the error due to the liberation of free ammonia at the pH of the end-point used. However, the urine occasionally showed more titratable acid plus ammonia than phosphate plus organic acids. This indicates that a certain proportion of the ammonium ions in the urine may be associated with the ions of strong acids, such as Cl^- or SO_4^{--} . This, however, does not invalidate our calculations inasmuch as it is feasible to assume a negative loss of base. We have used the term "loss of base" in a restricted sense, referring to the base in the urine which was derived from the buffer salts of the blood and body fluid.

Since it was the purpose of the experiment to determine the effect of diuresis on the alkali reserve of the blood, it was necessary to correlate the loss of base in the urine with the fall in alkali reserve of the blood. The method of calculation by which this was done is as follows: Total acidity was taken to be equivalent to the titratable acid (corrected) plus ammonia. The total base lost (as alkali salts of phosphoric and organic acids) in the urine represents the difference between phosphates plus organic acids and total acidity. The results were all expressed in terms of 0.1 molar or 0.1 normal concentration. The base lost in the urine and the alkali reserve were determined for a control period of 24 hours. After the 4 hour period of diuresis following injection, the base lost in the urine (over and above that excreted during the similar control period interval), and the alkali reserve were determined. The

percentage fall in alkali reserve was calculated. Assuming the body fluids of the dog to constitute 70 per cent of the total body weight, the calculations involved may be outlined in the following manner.

Dog 2, female, weight 10.7 kilos, see Table I.

Titratable acid (uncorrected).....	139.5 cc. of 0.1 N
“ “ (corrected).....	104.1 “ “ 0.1 “
Ammonia.....	159.1 “ “ 0.1 “

Total acidity.....263.2 cc. of 0.1 N

Phosphates.....	100.9 “ “ 0.1 M
Organic acids.....	190.8 “ “ 0.1 N

Total291.7 cc. of 0.1 N

“ acidity.....263.2 “ “ 0.1 “

Base lost in the urine..... 28.5 cc. of 0.1 N

The above is for the 24 hour control period.

4 hour diuresis period.

Titratable acidity.....	21.92 cc. of 0.1 N
“ “ (corrected).....	19.20 “ “ 0.1 “
Ammonia.....	43.80 “ “ 0.1 “

Total acidity.....63.00 cc. of 0.1 N

Phosphates.....	22.10 “ “ 0.1 M
Organic acids.....	109.60 “ “ 0.1 N

Total.....131.70 cc. of 0.1 N

“ acidity.....63.00 “ “ 0.1 “

Base lost.....68.70 cc. of 0.1 N

Base lost in 4 hour control period..... 5.70 “ “ 0.1 N

“ “ “ 4 “ experimental period in excess of 4 hour control period.....63.00 cc. of 0.1 N

CO ₂ -combining power at beginning.....	49.47 vol. per cent
“ “ “ end of 4 hours.....	41.92 “ “ “

Fall in CO₂..... 7.55 vol. per cent

Percentage fall in alkali reserve $\frac{7.55}{49.47} \times 100 = 15.2$ per cent

Weight of dog at beginning of experiment.....	10,700 gm.
Body fluids.....	70 per cent
Volume of body fluids.....	7,490 cc.
The body fluids are considered as containing the equivalent of 0.05 N available base.	

According to the fall in alkali reserve, the animal lost $7,490 \times 0.152 = 1,138$ cc. of 0.05 N base from the body fluids. But the actual loss of base in the urine is 63 cc. of 0.1 N base or 126 cc. of 0.05 N base.

$\frac{126}{1,138} = 0.1107 = 11.07$ per cent, the amount of the actual fall in alkali reserve accounted for in the urine. Assuming that the blood is $\frac{1}{11}$ of the total body weight, the percentage of base lost may be calculated on the basis of the blood.

$\frac{10,700}{11} = 972$ gm. of blood, approximately 972 cc. Percentage calculation of total base lost from the blood.

$972 \times 0.152 = 147.74$ cc. of 0.05 N base lost. 0.05 N base accounted for in the urine 126 cc.

$\frac{126}{147.74} = 0.853 = 85.3$ per cent.

Justification for this type of calculation is to be found in an examination of the various salts used to produce diuresis and those salts normally excreted. Disodium phosphate may be considered as the sodium salt of the weak acid, monosodium phosphate, and the sodium salts of the organic acids eliminated are also salts of relatively weak acids. The kidney has the ability to dissociate these salts of weak acids, of retaining the basic ions, and of eliminating monosodium phosphate and free organic acids. However, sodium sulfate, sodium chloride, and sodium nitrate are all salts of strong acids and the kidney is not capable of retaining the basic ions of these salts. Therefore, these neutral salts are eliminated as such. In being eliminated, these neutral salts retain water, due to an osmotic resistance to reabsorption. Consequently, the tubules are flooded, and reabsorption is less complete. As a result, it becomes impossible for the basic ions of the salts of weak acids present to be extensively reabsorbed and the base is lost, where normally it would be retained. The titratable acidity plus ammonia has, for a long time, been taken as a measure of the acid production in metabolism. The difference, therefore, between the titratable acidity plus ammonia and the phosphates plus organic acids must be a measure of the base lost from the body, either in

the form of dibasic phosphate or salts of organic acids. Therefore, during diuresis, when the kidney is not able to retain the basic ions efficiently, this difference must increase, since more and more of the phosphates and organic acids, normally eliminated in the acid or free form, are excreted in the salt or basic form. It is permissible, therefore, to regard the increase in the difference between the phosphates plus organic acids and the titratable acid plus ammonia as an accurate index of the amount of phosphate or organic acids present in the salt form. From this, the loss of base due to diuresis may be calculated.

DISCUSSION.

In all cases, it was found that diuresis produced by intravenous injections of strongly hypertonic solutions of sodium nitrate, sodium chloride, sodium sulfate, and urea, caused a more or less marked fall in alkali reserve of the blood, a rise in the pH of the urine, and a considerable loss of base through the kidney in the form of basic phosphates and the salts of organic acids. In many cases, it was not possible to show a close agreement between the loss of base through the kidney and the loss calculated from the decline in the alkali reserve of the blood. This is not surprising, inasmuch as the equilibrium between the blood and the tissues may not have been established at the time when the samples of blood were drawn for analysis. This was undoubtedly the case in the results obtained 4 hours after the injection. Furthermore the assumption that the body fluids of dogs are 0.05 N solutions of available base cannot be more than approximately true.

In Dogs 2, 3, 7, and 15, the loss of base through the kidney, and that calculated from the change in the CO_2 -combining power of the blood were in rather close agreement. In the case of Dog 2, Tables I and II, the urine was increased from 112 cc. in the control 24 hours to 685 cc. in the 4 hour experimental period. Along with this increase in urinary output, the base lost in the urine was 126 cc. of 0.05 N solution in excess of a similar period on the control day. At the end of the 24 hour experimental period, however, the base lost in excess of the control day had increased to 583 cc. of 0.05 N solution. The calculated base lost from the body fluids, basing the calculations on the percentage fall in the alkali reserve,,

TABLE I.

Dog No.	Base in urine in excess of control period, 0.1 N.	Fall in alkali reserve.	Base lost from body fluids accounted for in urine.	Base lost from blood accounted for in urine.	Remarks.
	<i>cc.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
1	64.9 201.8	9.12 -7.47	14.4	111.0	Short period. 24 hr. "
2	63.0 281.5	15.2 8.5	11.1 91.5	85.3 707.0	Short " 24 hr. "
3	334.3 509.8	20.6 4.43	30.9 219.0	227.0 1680.0	Short " 24 hr. "
4	136.6 104.2	15.9 8.2	17.9 25.2	115.2 201.4	Short " 24 hr. "
5	56.1 58.8	6.4 -1.51	15.9	122.7	Short " 24 hr. "
6	-3.7 -76.2	15.4 0.26			Short " 24 hr. "
7	18.4 230.4	19.5 4.25	1.7 98.5	13.2 757.8	Short " 24 hr. "
8	89.3 -55.3	4.5 -20.3	27.9	215.2	Short " 24 hr. "
9	23.4 101.4	13.2 -8.7	3.5	26.6	Short " 24 hr. "
10	31.5	25.5	5.6	41.7	Short " Dog died in night.
11	17.8 68.8	6.37 7.6	6.7 23.2	57.7 177.5	Short period. 24 hr. "
12	21.8 25.1	-17.97			Short " 24 hr. "
13	65.7 156.8	Too small to calculate.			Short period. 24 hr. "
		6.28	90.5	695.0	
14	84.0 121.5	7.17 17.1	32.2 22.6	247.8 159.5	Short " 24 hr. "

TABLE I—*Concluded.*

Dog No.	Base in urine in excess of control period, 0.1 N.	Fall in alkali reserve.	Base lost from body fluids accounted for in urine.	Base lost from blood accounted for in urine.	Remarks.
	cc.	per cent	per cent	per cent	
15	104.1	0.38			Short period.
	211.9	4.71	77.4	596.2	24 hr. “
	199.6	5.33	68.6	528.0	48 hr. “
					This was calculated on weight (15.6 kg.) at at end of experiment.
16	147.2	8.44	47.7	367.1	Short period.
	211.0	9.45	61.1	470.1	24 hr. “
17	58.7	2.69	34.9	260.9	Short “
	-18.0	-5.51			24 hr. “
18	324.2	10.94	54.1	417.0	Short “
	314.5	-9.84			24 hr. “
20	208.5	6.54	84.0	646.7	Short “
	279.1	-1.08			24 hr. “
21	150.3	0.53			Short “
	204.4	-2.65			24 hr. “

was equal to 637 cc. of 0.05 N alkali. Therefore, 91.5 per cent of the calculated base lost was accounted for in the urine. In the case of Dog 7, Table I, at the end of the 24 hour period, the agreement was even closer than in the previous case, since, of the calculated base lost from the body fluids, 98.5 per cent was accounted for in the urine, by an increased base elimination. In every case except that of Dog 6, Tables I and II, there was a loss of base in the urine during the period of diuresis over and above the amount eliminated on the control day. This case will be discussed later. Concomitant with this loss, the alkali reserve fell, sometimes only slightly; but in Dogs 2 and 7, Table I, the alkali reserve was 20.6 and 19 per cent, respectively, lower than on the control days. Usually, in the 20 hour period following extreme diuresis, there was a tendency of the alkali reserve to return somewhat toward nor-

TABLE II.

Date.	Time.	Blood analyses.		Urine analyses.					Remarks.		
		pH	Alkali reserve CO ₂ .	Oxygen capacity.	0.1 N acid.	0.1 N ammonia.	pH	0.1 N phosphates.		0.1 N organic acids.	Phosphate.
					cc.	cc.		cc.	cc.		cc.
Dog 1, female, weight 14.12 kilos.											
1924			vols. per cent	vols. per cent							
Nov. 17	8.00 a.m.	43.30			244.2	359.6	5.8	178.8	373.9	0.47	370
	1.50 p.m.	7.29	39.35		57.2	109.2	6.36	40.9	171.6	0.26	650
" 18	9.10 a.m.	50.77			134.0	108.0	6.38	163.0	128.0	1.31	1000
Dog 2, female, weight 10.7 kilos.											
Nov. 19	9.00 a.m.	7.32	49.47		139.5	159.1	5.95	100.9	190.6	0.53	112
	2.05 p.m.	7.23	41.92		21.9	43.8	6.95	22.1	109.6	0.21	685
" 20	9.00 a.m.	7.28	45.23		52.8	86.4	6.85	133.4	153.6	0.87	400
Dog 3, female, weight 15 kilos.											
Dec. 1	10.00 a.m.	7.33	48.66		663.1	328.2	5.60	584.0	304.5	1.90	540
	2.05 p.m.	7.31	38.91		466.3	141.4	6.35	423.3	485.5	0.87	1360
" 2	9.10 a.m.	7.45	46.51		228.8	36.4	6.10	177.7	166.4	1.06	650
Injected 180 cc. 10 per cent NaNO ₃ at 10.45-10.55 a.m. P:O whole period is 0.77.											
Injected 150 cc. 10 per cent NaNO ₃ at 10.45-10.55 a.m. P:O whole period is 0.60.											
Injected 200 cc. 10 per cent NaNO ₃ at 10.00-10.10 a.m. P:O whole period is 0.92.											

Dog 4, female, weight 13.64 kilos.

Dec. 8	9.00 a.m.	7.42	48.13	228.5	178.2	6.20	148.4	348.2	0.43	340	Injected 200 cc. 10 per cent NaNO ₃ at 10.18-10.23 a.m. P:O whole period is 0.60.
" 9	2.05 p.m.	7.38	40.47	54.6	33.6	6.95	37.6	193.2	0.199	1050	
	9.10 a.m.		44.18	198.0	121.0	6.80	194.0	180.4	1.05	550	

Dog 5, female, weight 15.70 kilos.

1925											
Jan. 7	10.30 a.m.	7.36	52.34	199.8	58.1	5.80	106.7	221.5	0.48	110	Injected 240 cc. 10 per cent NaNO ₃ at 10.30-10.40 a.m. P:O whole period is 0.995.
" 8	2.00 p.m.	7.30	49.00	31.36	23.5	6.80	23.3	101.9	0.22	980	
	9.10 a.m.	7.37	53.85	264.0	77.4	5.60	231.3	154.9	1.49	440	

Dog 6, female, weight 13.82 kilos.

Jan. 14	9.00 a.m.	7.46	57.22	218.9	62.56	6.60	231.8	362.8	0.64	460	Injected 250 cc. 10 per cent NaNO ₃ at 10.30-10.40 a.m. P:O whole period is 1.04.
" 15	2.15 p.m.	7.43	48.41	31.96	18.7	6.85	43.4	82.7	0.52	940	
	9.00 a.m.	7.46	57.07	231.0	43.1	6.24	241.8	120.1	2.01	385	

Dog 7, female, weight 10.7 kilos.

Jan. 29	9.30 a.m.	7.45	51.73	272.2	188.2	6.10	206.6	322.6	0.64	420	Injected 150 cc. 10 per cent NaNO ₃ at 10.45-10.55 a.m. P:O whole period is 0.65.
" 30	2.00 p.m.	7.44	41.72	25.0	41.6	6.95	11.6	83.2	0.14	1040	
	9.00 a.m.	7.35	49.53	114.8	59.4	6.85	205.8	237.6	0.87	330	

Dog 8, female, weight 20.2 kilos.

Feb. 2	9.50 a.m.	7.46	42.93	224.0	151.2	5.90	130.4	344.0	0.39	200	Injected 265 cc. 10 per cent NaNO ₃ at 10.00-10.15 a.m. P:O whole period is 0.60.
" 3	2.00 p.m.	7.48	40.99	61.9	113.5	6.90	65.1	206.4	0.31	1720	
	9.45 a.m.	7.48	51.65	278.6	220.3	5.60	194.1	228.9	0.85	540	

TABLE II—Continued.

Date.	Time.	Blood analyses.			Urine analyses.						Remarks.		
		pH	Alkali reserve CO ₂	Oxygen capac- ity.	0.1 N acid.	0.1 N ammonia.	pH	0.1 N phos- phates.	0.1 N organic acids.	Phosphate.			
										Organic acid.		Volume.	
Dog 9, female, weight 14.6 kilos.													
1925			vols. per cent	vols. per cent	cc.	cc.		cc.	cc.		cc.		
Feb. 6	10.00 a.m.	7.47	49.63	25.82	123.1	81.3	6.25	113.2	136.2	0.83	460	Injected 265 cc. 10 per cent NaNO ₃ at 10.00-10.15 a.m.	
"	2.00 p.m.	7.29	43.07	29.79	28.2	56.9	6.98	13.4	102.4	0.13	940	P:O whole period is 0.96.	
"	9.15 a.m.	7.46	53.97	25.47	203.5	81.4	6.40	218.4	138.1	1.57	385		
Dog 10, female, weight 6.5 kilos.													
Feb. 9	10.00 a.m.	7.46	51.53	21.05	196.2	103.3	5.65	181.6	122.4	1.48	340	Injected 125 cc. 10 per cent NaNO ₃ at 10.00-10.10 a.m.	
	2.00 p.m.	7.25	38.35	20.94	10.6	12.4	6.85	3.86	62.7	0.06	460	Dog died during night.	
Dog 11, female, weight 11.9 kilos.													
Feb. 11	10.00 a.m.	7.43	55.65	19.25	249.2	198.4	6.70	174.5	284.8	0.61	310	Injected 185 cc. 12 per cent urea at 10.00-10.10 a.m. Hemoglobinuria.	
"	1.30 p.m.	7.43	52.10	20.22	24.0	35.2	7.60	3.22	76.8	0.043	400	P:O whole period is 0.62.	
"	9.30 a.m.	7.45	51.43	23.94	118.6	46.8	6.50	115.2	111.2	1.035	260		
Dog 12, female, weight 14.46 kilos.													
Feb. 16	10.00 a.m.	7.45	51.35	15.39	233.8	154.0	6.25	153.1	224.0	0.68	175	Injected 200 cc. 12 per cent urea at 10.00-10.10 a.m.	
"	2.00 p.m.	7.49	51.73	17.91	38.7	22.9	6.95	25.2	54.0	0.54	450		

Dog 13, female, weight 7.87 kilos.

Feb. 20	10.00 a.m.	7.40	52.40	18.88	80.5	45.1	6.85	80.5	113.5	0.70	110	Injected 125 cc. 13 per cent Na_2SO_4 at 10.00-10.15 a.m.
"	2.00 p.m.	7.47	53.41	18.73	15.6	13.2	7.00	9.67	90.0	0.10	600	P:O whole period is 0.538.
"	9.00 a.m.	7.47	49.11	20.10	70.0	30.2	6.80	86.8	83.5	1.04	120	

Dog 14, female, weight 10.40 kilos.

Feb. 24	9.30 a.m.	7.50	50.51	21.70	169.0	33.0	5.80	114.9	140.0	0.82	250	Injected 225 cc. 8 per cent NaCl at 9.30-9.40 a.m.
"	2.00 p.m.	7.52	46.92	20.33	59.2	33.1	6.95	55.6	125.3	0.44	1740	P:O whole period is 0.71.
"	9.15 a.m.	7.25	44.10	21.31	105.8	53.3	6.85	118.1	115.2	1.02	550	

Dog 15, female, weight 16.6 kilos.

Feb. 26	9.30 a.m.	7.43	52.01	19.31	227.8	119.0	5.90	146.5	199.7	0.73	160	Injected 300 cc. 8 per cent NaCl at 9.30-9.40 a.m.
"	2.00 p.m.	7.32	51.81	19.67	55.2	38.3	7.55	21.4	176.6	0.12	1840	P:O 24 hr. period is 0.569.
"	9.15 a.m.	7.41	49.56	20.94	172.3	93.4	6.65	187.3	189.8	0.94	650	
"	9.00 "	7.37	49.28	22.12	207.0	162.0	5.92	85.8	262.8	0.33	225	

Dog 16, female, weight 10.45 kilos.

Mar. 2	9.30 a.m.	7.43	50.66	22.14	136.8	63.8	5.90	90.7	106.4	0.85	95	Injected 225 cc. 8 per cent NaCl at 9.30-9.50 a.m.
"	1.30 p.m.	7.45	46.39	19.83	55.4	22.2	7.30	32.1	184.8	0.17	1540	P:O whole period is 0.61
"	9.30 a.m.	7.45	45.87	20.85	129.8	60.0	6.98	144.2	103.4	1.40	550	

Dog 17, female, weight 18.4 kilos.

Mar. 4	9.30 a.m.	7.46	51.57	21.95	224.4	112.2	6.20	156.4	224.4	0.69	330	Injected 200 cc. 10 per cent NaNO_3 at 9.40-9.50 a.m.
"	2.00 p.m.	7.50	50.18	21.28	67.1	40.0	7.20	36.7	134.2	0.27	1190	P:O whole period is 0.77.
"	10.00 a.m.	7.45	57.08	23.45*	253.3	120.6	5.80	180.8	147.4	1.22	335	

* This result is probably incorrect.

TABLE II—*Concluded.*

Date.	Time.	Blood analyses.			Urine analysis.						Remarks.	
		pH	Alkali reserve	Oxygen capacity.	0.1 N acid.	0.1 N ammonia.	pH	0.1 N phos- phates.	0.1 N organic acids.	Phosphate.		Volume.
Dog 18, female, weight 15.64 kilos.												
1925												
Mar. 6	10.00 a.m.	7.43	51.90	20.61	134.0	102.0	6.40	82.7	176.0	0.47	305	Injected 300 cc. 10 per cent NaNO ₃ at 10.00-10.10 a.m.
"	2.00 p.m.	7.43	46.62	22.27	64.8	31.7	7.10	20.2	403.2	0.05	3600	P:O whole period is 0.475.
"	10.00 a.m.	7.41	55.91	21.07	292.4	94.6	6.00	243.5	151.4	1.61	860	
Dog 20, female, weight 10.85 kilos. (Fed 100 gm. of Klim during the 48 hr. period.)												
Mar. 11	9.30 a.m.	7.48	55.38	19.22	153.9	169.5	5.70	95.5	149.8	0.64	520	Injected 110 cc. 10 per cent NaNO ₃ at 9.35-9.50 a.m.
"	2.00 p.m.	7.45	51.76	19.03	49.4	23.1	7.00	37.3	230.7	0.16	2060	P:O whole period is 0.46.
"	9.30 a.m.	7.49	55.98	20.40	110.0	68.0	6.20	110.0	92.0	1.20	295	
Dog 21, female, weight 10.31 kilos. (Fed 100 gm. of Klim during the 48 hr. period.)												
Mar. 13	10.00 a.m.	7.40	46.78	19.89	135.2	126.9	6.00	83.2	128.9	0.64	520	Injected 130 cc. 10 per cent NaNO ₃ at 10.00-10.10 a.m.
"	2.00 p.m.	46.53	19.87	68.9	49.2	6.98	50.6	206.6	0.24	1230	P:O whole period is 0.33.	
"	10.00 a.m.	7.47	49.43	19.85	150.1	163.5	6.20	104.1	251.9	0.41	670	Urine contaminated with feces.

mal, the urine becoming less basic. In several experiments (Dogs 8, 12, 17, 18, 20, and 21), the alkali reserve even exceeded the control value.

However, a return to normal did not always occur. In Dogs 11, 13, 14, 15, and 16, the alkali reserve was lower at the end of the 24 hour experimental period than at the end of the 4 hour period. In these experiments, sodium chloride was the diuretic used, and it was found that diuretic effect of this substance was more prolonged and profound than that brought about by sodium nitrate. Where sodium nitrate was used, the diuresis passed off in about 6 hours, this being accompanied by a partial recovery of the normal acid-base equilibrium. On the other hand, where sodium chloride was used as the diuretic, the base was found still to be eliminated in excess of the control period at the end of the 24 hour experimental period. In the case of Dog 15, even after a 48 hour period following injection, the alkali reserve had fallen slightly below the value for the 24 hour experimental period. The base excreted in excess of the normal amount was also higher for the second 24 hour experimental period. The phenomenon of delayed fall in alkali reserve is very well illustrated in this case.

The animals received no food during the control or diuresis period, with the exception of Dog 6, Tables I and II, where the dog was fed bones accidentally on the control day. This caused an excessive amount of phosphates to be eliminated, with the result that the base lost on the control day was in excess of that lost during the diuresis period. Due to fasting, there may be a slight fall in the alkali reserve. This possibly was not taken into account in the experiments previous to those done on Dogs 20 and 21, Tables I and II. To eliminate this factor, these dogs were fed 100 gm. of Klim, a powdered milk preparation (equivalent to about 300 calories per day), over a period of 48 hours. As is apparent from the data on these dogs, Tables I and II, the same results were obtained as in those experiments where the dogs were fasted. There was a fall in the alkali reserve in Dog 20, Tables I and II, of 6.54 per cent, 85.95 per cent of which was accounted for by the increase of base in the urine. The results in Dog 21 were not as consistent. Suffice it to say, however, that the fall in alkali reserve was due to the diuresis and not to fasting. It may be well to remark that in fasting, dogs are not prone to produce acetone

bodies, and thus the fall in alkali reserve due to fasting is very slight during a fasting period of only 48 hours.

In nearly all of the experiments, the agreement between the base lost in the urine and the fall in alkali reserve was closest at the end of the 24 hour experimental period, the exceptions being where the alkali reserve returned to normal or exceeded the value for the control period. In using the strongly hypertonic salt solutions as diuretics, a decided shift in the distribution of fluids between the blood and the tissues occurs, the tissue fluids entering the blood stream to dilute the salts, in order to restore osmotic equilibrium. However, the acid-base equilibrium is nearly reestablished at the end of the 24 hour experimental period, the loss of base being evenly distributed between the blood and the tissues. The sample of blood drawn at this time for analysis, therefore, would be a more accurate index of the acid-base balance in all of the body fluids than that drawn at the end of the 4 hour diuresis period. This factor would tend to bring the base lost in the urine and the fall in alkali reserve into closer agreement at the end of the 24 hour period. It may be pointed out that there was no great dilution of the blood by the fluids from the body tissues. This is shown by the fact that the oxygen-combining power of the blood, in those cases in which this was determined, did not show a marked change. This shows also, that the changes in CO_2 -combining power of the blood were not due, to any great extent at least, to the concentration or dilution of the blood. The formation of the urine appears to have been sufficiently rapid to maintain a concentration of the blood at a relatively constant level.

As pointed out by Cushny (11), the total elimination of phosphates during the diuretic period tends to increase in absolute amount, while the percentage concentration falls. In the present work, Cushny's observations have been confirmed in most cases. However, the organic acid elimination increased markedly during the period of extreme diuresis (the first 4 hours). The fluids from the tissues pass into the blood after an injection of hypertonic salt solution. These fluids have been in contact with metabolically active cells. Hence, organic acids, such as lactic acid, would be carried into the blood stream. Lactic acid is a no-threshold substance, and therefore would be eliminated by the kidney,

together with its basic ions, by which it was buffered in the blood. This would cause a removal of salts of organic acids, accounting in some measure for the loss of base. Similar observations were made by Hendrix and Bodansky (4), where along with the fall in alkali reserve in early experimental uranium nephritis, the organic acids eliminated increased considerably, and the urine became more basic. For the 24 hour experimental period, the phosphate:organic acid ratio (P:O) (see Table II) was very close to that for the control 24 hour period. This would tend to nullify the possible contention that the increased excretion of organic acids might be due to an increased production of organic acids as in acidosis. Although the ratio drops during the first 4 hours (*i.e.*, the relative amount of organic acids increases), it usually returns to a value above normal for the following 20 hour period, and when the total phosphates and organic acids for the whole experimental period of 24 hours are considered, the argument is against a fall in CO₂-combining power due to an increased production of organic acids.

No evidence is available to establish definitely the nature of the basic ions, the elimination of which causes the drop in the alkali reserve. Naturally, the sodium ions would be eliminated in the greatest abundance, since they are normally present in combination with the phosphates and organic acids in the body fluids, and are present as bicarbonates in the blood plasma, ready to react with any acids which might find their way into the blood stream. It was observed, however, that on addition of oxalate solution to the diuretic urine, in the determination of titratable acidity, the precipitation of calcium as the oxalate was relatively heavier than the normal urine. However, this observation is only qualitative in nature. The increase in the amount of precipitate may have been due more to the character of the urine than to an increased concentration of calcium in solution.

SUMMARY.

1. It has been demonstrated that in diuresis due to sodium nitrate, sodium chloride, sodium sulfate, and urea, there is a loss of base through the kidney over and above that lost normally.
2. Together with this loss of base in the urine, there is a marked fall in the alkali reserve:

3. The loss of base is believed to be due to a failure of reabsorption from the tubules, since the tubules are flooded and overtaxed, and cannot be supposed to function as efficiently as normally in the retention of the basic ions.

4. The effect of sodium nitrate diuresis on the alkali reserve is transient, passing off quickly, while the effect of sodium chloride diuresis is more prolonged.

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ON THE CATALYTICALLY ACTIVE AND INACTIVE FORMS OF FERRIC OXIDE.

BY LARS A. WELO AND OSKAR BAUDISCH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The material presented in this paper represents the results of a part of the work we have been doing in the general field of the biological action of iron. We have been concerned particularly with those cases in which the presence of iron in some form is an aid to the reactions. In other words, we have been concerned with those biological or chemical reactions in which iron or some compound of it plays the part of a catalyzer. As examples we may mention: (1) the recent use (1) of broth plus the complex iron salt $\left[\begin{array}{c} \text{OH}_2 \\ \text{Fe} \\ (\text{CN})_5 \end{array} \right] \text{Na}_3$ instead of broth plus blood in the growth of *Bacterium leprosepticum*; (2) the use of ferrous hydroxide in the oxidation of lactic acid to pyruvic acid; and (3) the use of ferrous hydroxide and oxygen in the reduction of potassium nitrate (2). From ferrous hydroxide it is natural to proceed to the oxides of iron since ferrous hydroxide is itself converted to an oxide whenever it is used as a catalyzer in reactions involving the use of oxygen.

That certain oxides of iron function as catalyzers is well known, but beyond a knowledge of the fact that they do so function, we are very much in the dark. It must be confessed that with our work as it now stands the fundamental nature of iron catalysis is still open for further research. But we believe that with the data presented in the following discussion we have established the criteria which determine whether an iron oxide is or is not able to function as a catalyzer. It will be shown: (1) that, as is to be expected, the catalytic activity depends on the average size of the crystals; (2) that the catalytic activity depends on the

structure of the crystals composing the material; and (3) that the activity may *vanish entirely* as the *structure* of the crystals is changed from one form to another.

The Oxides and Their Derived Forms.

In this work we have always started out with the artificial magnetite Fe_3O_4 which may be written $\text{FeO} \cdot \text{Fe}_2\text{O}_3$ in order to bring out its mixed character, as it has both ferrous and ferric iron ions. Owing to a tendency to autoxidize, it is very difficult to obtain the exact proportion of ferrous iron to ferric iron as given in the formula $\text{FeO} \cdot \text{Fe}_2\text{O}_3$. In practice one always finds a smaller proportion of ferrous iron than the theoretical.

There are many ways of preparing magnetite. We find that the oxides yielded by the several methods fall in two groups. One group is represented by Lefort's oxide (3) which is the brownish black microcrystalline substance formed when a solution containing 1 mol of ferrous sulfate and 2 mols of ferric sulfate is poured into a boiling solution of sodium hydroxide. It is necessary that sodium hydroxide be present in excess so as to give, always, an alkaline solution. The precipitate is washed until the filtrate shows no alkali when tested with litmus paper and it is then collected on a filter and dried in a desiccator. It is noted that in this method of making magnetite the proportion of the ferrous and the ferric iron ions present *previous* to precipitation is 1:2 as it should occur in magnetite.

In the other group which we here call "mol-oxide" we shall place all of those magnetites which are made by the precipitation of ferrous hydroxide alone with no ferric iron present in any form. This procedure makes it necessary to oxidize at least two-thirds of the iron to the ferric form *during* or *after* the precipitation, by *molecular* oxygen from the air or from some chemical source of oxygen. It is for this reason that the term "mol-oxide" is used. In the main we have used two of the available methods for oxides of this type. One is that of Kaufmann (4). To 220 cc. of a 10 per cent solution of crystalline ferrous sulfate are added 22 gm. of a 20 per cent solution of ammonia and the mixture is boiled. During the boiling there are poured in, slowly, 2.55 gm. of potassium nitrate which has been dissolved in as little water as possible. The boiling is continued for 15 minutes. The

material, which settles rapidly, is easily decanted and washed and is then collected on a filter and dried. We may mention that the decantation can be hurried by holding the containing vessel over a large electromagnet. The other method involves the reaction first studied by Baudisch and Mayer (5) in which potassium nitrate is reduced by freshly precipitated ferrous hydroxide if oxygen is present in the solution. We (2) have recently studied this reaction more closely. As a by-product a magnetic oxide is formed which, we are now convinced, is also magnetite. A solution of sodium hydroxide and potassium nitrate is made up in water which *must* contain oxygen in solution. Into this is poured a solution containing sufficient ferrous sulfate to be equivalent to the sodium hydroxide. The proportions we have usually used of sodium hydroxide, potassium nitrate, and crystalline ferrous sulfate have been 2.6, 20, and 9 gm., respectively. This material settles rapidly, especially with the aid of the magnet, and is easily decanted and washed. It is then collected and dried.

Both of the oxides we have described, Lefort's and the mol-oxide, are magnetites having both divalent and trivalent iron. It is not generally known that artificial magnetite can be completely oxidized so that all of the iron is of the ferric form and that the oxide may yet stay fully as ferromagnetic as the original magnetite. Although this fact was first discovered in 1859 there are few references in the literature (6). It is generally supposed that the ferromagnetic properties of magnetite are due to the presence of ferrous iron. We (7) have recently shown that the crystal structure as well as the magnetic properties of magnetite are retained after the ferrous iron in it has all been converted to trivalent iron. We found that the oxidized magnetite became non-magnetic and changed its crystal structure only when heated to some higher temperature and that this change took place without a change in composition. This is a consequence of the fact that the ferric oxide Fe_2O_3 has allotropic forms.

It is clear that the transformation of the magnetite into the non-magnetic ferric oxide Fe_2O_3 , hematite, takes place in two distinct stages. It is also clear that because of this property, we can derive from magnetite two distinct oxides; one being the magnetic ferric oxide of composition Fe_2O_3 which we may call oxidized magnetite, and the other is the non-magnetic ferric oxide of the same composition and is the usual hematite.

These derived oxides were obtained from both groups of magnetite, Lefort's and the mol-oxide. The oxidized magnetite is obtained by heating magnetite at a temperature of about 330°C. in a stream of oxygen for an hour or two. The non-magnetic ferric oxide is obtained by heating the oxidized magnetite to 550°C. or more. This latter heating need not be made in the presence of oxygen if the previous oxidation be complete. This temperature of transformation is somewhat variable from sample to sample so that it is best to heat to a glow to insure that the transformation is complete.

Indicators of Catalytic Activity.

As indicators of the catalytic activity of the oxides we have used three reactions. They are: (1) the ordinary blood test; (2) the influence on bacterial growth; and (3) the absorption of oxygen.

The blood test consists, as is well known, in the oxidation of freshly dissolved benzidine chloride by the atomic oxygen set free by the dissociation of hydrogen peroxide. This dissociation and the yield of atomic oxygen is brought about by the hemoglobin of the blood and is indicated by the blue oxidation product of benzidine. This same blue color is obtained if, instead of blood, we use a small pinch of those oxides which are "active." If the "inactive" oxide is used for this test we get again a dissociation of hydrogen peroxide, but we do not obtain the blue color of a positive blood test. The oxygen set free is, in this case, molecular and cannot oxidize benzidine.

The influence on bacterial growth was studied with *Bacterium lepi-septicum*. Under aerobic conditions this bacterium does not thrive and soon loses its virulence if cultivated in broth alone. The bacterium does thrive and does retain its virulence if cultivated in broth plus blood. Likewise, it will thrive and stay virulent if grown in broth into which is dropped a pinch of those oxides which are "active." The technique and fuller discussion of this test is being given elsewhere (1).

The studies on bacterial growth suggested that the function of the oxide is, at least in part, that of absorbing oxygen so as to produce anaerobic conditions in the broth. We were thus led to examine their oxygen absorption while they were covered with broth or water. The absorption measurements were made for

us by Dr. J. A. Hawkins of this Institute by means of the Barcroft apparatus (8). On the basis of this oxygen absorption test, too, it was found that oxides are to be classed as "active" and "inactive" and that the classification corresponds to that obtained by the blood test and by the test for bacterial growth.

RESULTS.

The results given by the oxides, when tested with the three reactions we have mentioned, are summarized in Table I. This table shows also the results of other physical or chemical tests which are of interest or which have a direct bearing on the interpretation of the relative behavior of the oxides when used as catalyzers. The Prussian blue test for ferrous iron does not need description. The values for the magnetic susceptibilities are calculated from the permeabilities measured by a method we are describing elsewhere (9). The data on the crystal structures were obtained for us by Dr. Wheeler P. Davey of the General Electric Company, the diffraction patterns being compared with published data for magnetite and hematite. The water absorption is expressed as percentage of the dry weight. The absorption is from the saturated vapor such as exists in an evacuated desiccator at ordinary temperatures when the usual sulfuric acid drier is replaced by water.

The table shows us at once that the magnetite and the oxidized magnetite of the mol-oxide are very poor catalyzers as compared with the corresponding oxides of Lefort. A hint as to the reason is contained in the values for the water absorption; one absorbing, on the average, only a thirteenth of that absorbed by the other. This is readily understood if we recognize the fact that a given particle of the powdered oxide is an aggregate of extremely minute crystals. We may assume that the crystals as they appear in the oxides of Lefort are, on the whole, much smaller than the crystals of the mol-oxide, and thus give, to the Lefort oxides, a much larger area of crystal face in proportion to the mass of oxide. The effectiveness of the oxides as catalyzers should be affected in the same way as the water absorption. That this view is the right one is strikingly confirmed by the x-ray diffraction patterns. They are shown in Fig. 1. The system of lines is the same for both of the magnetites so that we are sure that the

TABLE I.

	Lefort's oxide.			Mol-oxide.		
	Fe ₃ O ₄ (Magnetite.)	Active Fe ₃ O ₄ , Fe ₃ O ₄ oxidized at 330°C. (Oxidized magnetite.)	Inactive Fe ₃ O ₄ , Fe ₃ O ₄ oxidized at 330°C., then heated to 550°C. (Hematite.)	Fe ₃ O ₄ (Magnetite.)	Active Fe ₃ O ₄ , Fe ₃ O ₄ oxidized at 330°C. (Oxidized magnetite.)	Inactive Fe ₃ O ₄ , Fe ₃ O ₄ oxidized at 330°C., then heated to 550°C. (Hematite.)
Prussian blue test for ferrous iron.....	+	—	—	+	—	—
Maximum magnetic susceptibility, χ . Non- magnetic if $\chi = 0$	0.152	0.182	0.0036	0.141	0.127	0.0016
Crystal unit.....	Cubic.	Cubic.	Rhombo- hedral.	Cubic.	Cubic.	Rhombo- hedral.
Absorption of water, average <i>per cent.</i>	27	27	21	2.1	2.0	0.5
Blood test. Oxidation of benzidine hydro- chloride by H ₂ O ₂ in presence of.....	Good.	Good.	None.	Fair.	None.	None.
Growth of <i>B. leprosepticum</i> in broth and in presence of.....	"	"	"	"	Slight or none.	"
Absorption of oxygen. Average initial rate, $\frac{\text{cm.}^3}{\text{gm. hr.}}$	0.0044	0.0080	0	0.00191	0.00048	0



FIG. 1.



FIG. 2.

atoms of iron and oxygen are arranged in the same way within the crystals. But it is seen that the lines given by Lefort's magnetite are diffuse whereas the lines given by the magnetite of the mol-oxide have sharp and clear-cut edges. This is exactly as it should be if the average crystal is small in the former case and large in the latter. It may be mentioned that this relation between the sharpness of the diffraction pattern lines and the sizes of the crystals is the basis of a method of estimating the average dimensions of very minute crystals (10). We have not made the measurements on the patterns which are necessary for an estimate of the sizes of the oxide crystals. It is sufficient for our purpose to know that the average crystal of the oxides of Lefort is small as compared with that of the mol-oxide and that the total area of exposed crystal face is correspondingly large in proportion to the mass of oxide. We need no longer, therefore, consider any of the data under the mol-oxide.

Let us now turn to a comparison of the three columns under Lefort's oxide. It is noted that the magnetite and oxidized magnetite are practically alike in all respects except one. The magnetite contains ferrous iron and gives the Prussian blue test. Oxidized magnetite does not. We have devoted much time to this interesting phenomenon, that the magnetic properties and the crystal structure remain unchanged during oxidation. They do, however, change at a higher temperature without a change in composition.

From the point of view of crystal structure it is particularly difficult to understand how the cubic structure of magnetite can remain stable and unchanged in spite of the oxidation of the ferrous iron to the divalent form. The fact of the matter, as developed by experiment and shown in the table, is that ferric oxide of cubic structure *is unstable* and takes on the more stable structure with a rhombohedral unit, but does not do so until a sufficiently high temperature is reached.

At any rate, the iron in oxidized magnetite gives none of the accepted tests for the presence of divalent iron. It gives no Prussian blue, there is no indication of an oxidation by potassium permanganate, and there is no increase in weight when oxidized magnetite is converted to hematite. It is extremely improbable that the magnetite should acquire by absorption, but without

chemical combination, exactly the right weight of oxygen to be converted to hematite on heating at the higher temperature.

We must conclude, then, in spite of certain difficulties, that there is no ferrous iron in oxidized magnetite. And we must likewise conclude, from a comparison of the data in the first two columns under Lefort's oxide, that the catalytic action of iron oxides *does not depend* on the presence of iron in the ferrous form.

That the catalytic action of iron oxide is dependent, directly or indirectly, on a particular arrangement of the atoms of iron and oxygen within the crystal is clearly indicated in the table. Reference is made to the second and third columns of the table under the heading, "Lefort's oxide." The data in the third column show that the ferric oxide made by oxidizing Fe_3O_4 at a low temperature and then heating it to 550°C . or more has no catalytic action whatsoever. Yet it is identical chemically to the active Fe_2O_3 . We have already considered the absence of ferrous iron from both of these oxides. The difference in catalytic activity cannot be due to the presence in the active Fe_2O_3 of an impurity which may have been driven off when it was transformed to the inactive Fe_2O_3 by heating at the higher temperature. Table II shows that, within the limits of accuracy of the analyses, the two oxides have the same percentage of Fe_2O_3 and an impurity which is very probably sodium sulfate.

This sodium sulfate is formed during the precipitation of the iron sulfates with sodium hydroxide and becomes lodged within the particles of the oxide so that it cannot be washed out.

Except for the rather small difference in water absorption which will be considered later, the only apparent differences between the oxides are in their magnetic susceptibilities and their crystal structures. Fig. 2 shows the x-ray diffraction patterns for the active and inactive ferric oxides along with the pattern given by the magnetite, Fe_3O_4 , from which the two ferric oxides were derived. Unfortunately the lines of the central undiffracted x-ray beam were cut off from the films during the preparation of this plate. It is seen that no changes occur in the pattern when magnetite is changed into the magnetic oxidized magnetite, active Fe_2O_3 . A different pattern is given by the practically non-magnetic and inactive Fe_2O_3 .

The arrangement of the iron and oxygen atoms in the crystals giving the patterns of Fig. 2 is shown in Figs. 4, 5, and 6 of our paper on the aging of ferrous hydroxide and ferrous carbonate (11).

Figs. 4 and 6 are from published data (12) and were not deduced by us from the patterns of Fig. 2. It was only necessary for us to see that these patterns correspond to the patterns which have led others to the atomic arrangements they have published. The patterns do correspond except for small and uncertain differences

TABLE II.

Oxide.	Sample No.	Fe ₂ O ₃	Impurity.
		<i>per cent</i>	<i>per cent</i>
Active Fe ₂ O ₃	1	97.0	3.0
“ “	2	96.2	3.8
Inactive “	1	97.6	2.4
“ “	2	97.0	3.0

TABLE III.

Oxide.	Temperature.	Time of transformation.	Water absorption (per cent of dry weight).
	<i>°C.</i>	<i>min.</i>	<i>per cent</i>
Active Fe ₂ O ₃			29.7
Inactive “	600	10	26.1
“ “	800	2	26.6
“ “	800	4	24.5
“ “	800	50	21.0

in spacing of the lines which are of the order of one-tenth of a per cent. Fig. 5 is from a paper published elsewhere (9) in which we deal with the problem of finding space for more oxygen in the magnetite structure so as to convert it to the ferric oxide, Fe₂O₃, structure without disturbing the arrangement of the atoms already in place.

The inactive ferric oxide absorbs less water than the active ferric oxide. This is explained by the fact that, during the transformation in crystal structure, the new crystals grow and present relatively less surface for contact with water vapor. Table III

affords an illustration of this growth of crystals (at sufficiently high temperatures) and the relation to the absorption of water. The absorption of water is, obviously, not selective and takes place with both oxides. The tests were made with an oxidized magnetite from Lefort's Fe_3O_4 .

In view of the fact that those mol-oxides which are active have a much smaller water absorption than the 21 per cent absorbed by the inactive Fe_2O_3 of Lefort, we cannot assume that the latter is inactive because the crystals have grown, during the transformation, to such sizes that the total area of crystal face becomes negligibly small. The smaller water absorption, as compared with the active Fe_2O_3 , does indicate crystal growth and decrease of the exposed crystal surface; but the decrease is far too small to account for the absence of activity. The activity of the oxides of iron as catalyzers is related in some way to the arrangement of the atoms in a cubic lattice and disappears entirely when the same atoms become arranged in the rhombohedral lattice of hematite.

The result of still another experiment¹ eliminates as a factor the possible growth of the crystals to such an extent that the total active crystal face becomes negligible as compared with the total crystal face in the active oxide. Ferric hydroxide, $\text{Fe}(\text{OH})_3$, was precipitated from solutions of equivalent parts of ferric sulfate and sodium hydroxide. On heating to a glow, dehydration took place and ferric oxide, Fe_2O_3 , was formed. This oxide did not give the benzidine or blood test although its water absorption from a saturated atmosphere was very high. This particular sample absorbed 97 per cent of its dry weight. A part of this water was free and flowed about in the boat in which the dry oxide had been placed.

DISCUSSION.

It would seem to be somewhat fanciful to ascribe the catalytic action of the active ferric oxide to its marked quality of ferromagnetism and to say that the inactive ferric oxide is inactive because it is non-magnetic. We are rather of the view that the properties of ferromagnetism and catalytic activity in iron oxides are *accompanying* properties which are both related to the arrange-

¹ The experiment was suggested by Dr. R. W. G. Wyckoff of the Geophysical Laboratory, Washington.

ment of the atoms in space, either directly or indirectly. It is not unlikely that when, if ever, we can really understand the phenomenon of polymorphism as it appears in ferric oxide we shall also gain a very real insight into the nature of ferromagnetism.

A more promising theory is to imagine that the "topography" of the small crystal face changes as the ferric iron oxide is transformed from the cubic structure to that having rhombohedral units. By "topography" we mean the appearance of the crystal surface as looked at from the outside and from the point of view of atomic dimensions. It is necessarily different in the two cases we are considering, since the atoms of iron and oxygen are differently arranged throughout the body of the crystal and right up to the surface. The free valence forces exhibited at the surfaces of the crystals would not be the same for the two cases.

It is possible that the orientations of the iron atoms, or of the oxygen atoms, or of both the iron and oxygen atoms would be distinct for the two oxides in question and that the individual atoms themselves present different faces towards the exterior. No matter which of these possibilities is chosen, this additional assumption would have to be made: in the active oxide certain linkages or activations take place, and they cannot take place with the inactive oxide because its surface forces are not distributed and directed in the right way.

The ultimate solution awaits a more complete knowledge of the atomic structures of iron and oxygen. Possibly, the appearance of two crystal forms of ferric oxide reveals to us the existence of two distinct arrangements of the complex electronic system which is recognized chemically as the trivalent atom of iron.

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THE DETERMINATION OF NITRATE NITROGEN IN PLANTS.

BY ROBIN C. BURRELL AND THOMAS G. PHILLIPS.

(From the Department of Agricultural Chemistry, the Ohio State University, Columbus.)

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The methods used for the determination of nitrate nitrogen in plant materials have been adapted from methods used in the analysis of soils and fertilizers. The one most used at present involves reduction by Devarda's alloy, and the determination by titration of the ammonia formed. This method has been studied in detail for soils by E. R. Allen (1), Davisson (2), and Whiting, Richmond, and Schoonover (3). The peculiar difficulties met in applying it to plant extracts, however, have not been investigated sufficiently. The object of the first part of this work was to learn whether nitrate nitrogen can be determined by reduction by Devarda's alloy in the presence of the other nitrogen compounds likely to occur in plant extracts.

The materials used were sodium nitrate, made by neutralizing a standard solution of c.p. nitric acid with sodium hydroxide; ammonium sulfate; asparagine; and alanine. These were of the highest purity obtainable and their composition was checked by analysis. The distillations were made in 500 cc. Pyrex Kjeldahl flasks through Davisson scrubber bulbs and block tin condensers. Methyl red was used as indicator and the solutions of sodium hydroxide and sulfuric acid were 0.02 N. Ammonia-free water was used wherever necessary.

Distilling from 1 gm. of Devarda's alloy and a volume of 300 cc. of approximately 0.1 N sodium hydroxide for 1 hour, gave recoveries from 2 mg. of nitrate nitrogen varying between 97.1 and 99.7 per cent, with an average for eight determinations of 98.4 per cent.

Attempts were made to recover nitrate nitrogen from mixtures containing 1 mg. each of nitrate nitrogen, ammonia nitrogen, and amide nitrogen (as asparagine), and 2 mg. of amino nitrogen (1 mg. as asparagine and 1 mg. as alanine). To remove nitrogen other than nitrate that might be evolved as ammonia during the alkaline reduction the methods of Davisson (2) (preliminary boiling with sodium hydroxide), and that of Whiting, Richmond, and Schoonover (3) (preliminary boiling with sodium peroxide), were used. The results obtained were not consistent and were usually high. It was suspected that the amide nitrogen of asparagine was not completely removed by the preliminary treatment, and continued to be evolved during the reduction. The Davisson method was carried out with samples containing no nitrate nitrogen but 2 mg. of amide nitrogen as asparagine, with yields of "nitrate nitrogen" varying from 0.28 to 0.31 mg. These methods for nitrate nitrogen, then, are not applicable in the presence of asparagine.

The comparison method as used by Strowd (4) was then tried. Two aliquots were distilled in the same way, except that Devarda's alloy was used in one of them. The difference in the amount of ammonia recovered was taken as due to the nitrate (and nitrite) nitrogen reduced. The recoveries from 1 mg. of nitrate nitrogen amounted to 75.9, 75.9, and 85.3 per cent. In order to learn which constituent of the solution interfered with the recovery of nitrate nitrogen, the following mixtures were analyzed. Each contained 1 mg. of nitrate nitrogen.

Other nitrogen present.		Recovery of nitrate nitrogen.
		<i>per cent</i>
1 mg. alanine	nitrogen.....	99.6
		99.7
1 "	ammonia "	91.1
		88.2
2 "	" "	89.9
		85.7
1 "	amide nitrogen	} as asparagine.....
1 "	amino acid nitrogen	
		(81.2
		72.7
		78.3
		75.7

The comparison method, then, is not applicable in the presence of ammonia nitrogen, and is especially faulty in the presence of amide nitrogen.

Because of the failure of the reduction method, and because it is often desirable to determine amounts of nitrate nitrogen much less than 1 mg., it was decided to attempt the application of a colorimetric method. Two methods seemed to give promise, the phenol disulfonic acid method, especially as applied to soils, and the modified reduced strychnine method (5).

The latter was soon discarded because of the off tints developed in the presence of nitrites and other compounds that are frequently present in plant extracts, and because of the rapid change of the color on exposure to light, which made quantitative comparisons difficult.

In attempting the application of the phenol disulfonic acid method, three principal difficulties were met: (1) clearing the plant extracts; (2) removing sugars and other substances that char on the addition of the reagent; and (3) overcoming the effect of chlorides.

Clearing the Plant Extracts.—The method is to be applied to the 80 per cent alcoholic extracts of fresh plant material. The alcohol is evaporated from an aliquot of such an extract and the residue taken up with water. Samples prepared in this way often are very highly colored. Preliminary tests of most of the usual protein precipitants and clarifiers showed charcoal and compounds of lead and copper to be most promising. These were studied further.

The use of copper sulfate, calcium hydroxide, and magnesium carbonate, as applied to soils by Harper (6), gave filtrates that were noticeably colored except when the extracts themselves were very light colored. In the case of some soil extracts, Harper used a preliminary treatment with a special charcoal. This was tried with plant extracts, but if they were very highly colored, two treatments with the charcoal were necessary, and on evaporation of these seemingly very clear solutions, finely divided charcoal began to separate and had to be removed by a copper hydroxide treatment. The charcoal residue from the clarification was frequently gummy. On adding known amounts of nitrates (0.1 mg. of nitrate nitrogen to 50 cc. of the extract), variable but con-

siderable losses were observed, amounting to from 10 to 20 per cent for each charcoal treatment, even when the charcoal residue was washed three times with 10 cc. portions of hot water.

Lead acetate solution alone would not give a clear filtrate. When 7.5 cc. of normal sodium hydroxide and 5 cc. of a 25 per cent lead acetate solution were added to 50 cc. of the extract prepared as described above, the desired result was obtained. The excess lead was removed by adding sulfuric acid.

Removing Substances That Char on Addition of the Reagent.—On adding the reagent to the residue from the evaporation of the clear filtrate obtained by the above treatment, there was always a slight charring which gave an off color to the final picrate solution, making an accurate comparison with the standard impossible. This was found to be due largely to the presence of small quantities of organic substances not removed by the lead acetate clarification. A fraction of a mg. of glucose, for instance, is sufficient to give a distinctly off color, even though the apparent charring is relatively slight. These disturbing substances must be removed. Direct oxidation of the whole plant extract by sodium peroxide was tried after removal of the alcohol by evaporation. This required considerable time and the quantity of salts, especially carbonates, in the final residue was so great that there was serious nitrate loss when the phenol disulfonic acid reagent was applied. A much smaller quantity of sodium peroxide was sufficient to oxidize the disturbing substances in the filtrate from the lead acetate clarification; and this procedure proved satisfactory.

Overcoming the Effects of Chlorides.—Enough chlorides are present in most plant material to prevent the quantitative determination of nitrates by the phenol disulfonic acid method. It is probable that the loss is due to the formation of *aqua regia* (7). In attempting to avoid this difficulty, the Gericke modification (8) was tried, but it gave very inconsistent results. It seemed necessary to remove the chlorides. Precipitation by silver sulfate was tried. The precipitate was, in part, of such a colloidal nature that it could not be removed by ordinary means. However, the use of copper sulfate, calcium hydroxide, and magnesium carbonate carried down the colloidal silver chloride along with the excess silver and the copper and gave a perfectly clear filtrate entirely free from interfering organic matter and chlorides.

RESULTS.

The method, as described below, was applied to the following solutions, with the results given in Table I.

TABLE I.

Solution A.—This contained 0.025 gm. of asparagine, 0.1 gm. of glycine, 0.01 gm. of ammonium sulfate, and 0.5 gm. of glucose per liter of aqueous solution.

Solution B.—The 80 per cent alcoholic extract of 50 gm. (fresh weight) pinnate leaves of 6 week old navy bean seedlings. Total volume, 1 liter.

Solution C.—Standard nitric acid exactly neutralized with sodium hydroxide and diluted so that each cc. contained 0.01 mg. of nitric nitrogen.

Quantity of solution used.	Nitric nitrogen added.	Total amount of nitric nitrogen found.	Range of recovery of added nitrate.	Average percentage of recovery of added nitrate.	No. of trials.
	mg.	mg.	mg.	per cent	
50 cc. Solution A.	0.00	0.00*			4
50 " " A.	0.10	0.097	0.096-0.098	97	2
50 " " A.	0.50	0.490	0.485-0.495	98	3
50 " " B.	0.00	0.825			2
50 " " B.	0.10	0.920	0.092-0.098	95	10
50 " " B.	0.50	1.305	0.47-0.50	96	3
50 " " C.	0.00	0.50		100	2

* Traces of nitrites.

(80%) *The Modified Method.*

An aliquot of the alcoholic extract of the sample (usually 50 cc., representing 2.5 gm. of the fresh leaf material) is evaporated on the steam bath until the alcohol is removed. Water is added to make the volume about 50 cc.; 7.5 cc. of normal sodium hydroxide and 5 cc. of 25 per cent lead acetate solution are added; and the mixture is stirred. The heavy precipitate is removed best by centrifuging, and washed twice in the centrifuge with 20 cc. portions of hot water. Excess lead is removed by adding to the clear liquid 0.5 cc. of concentrated sulfuric acid. The lead sulfate is filtered or centrifuged out, and washed once with a small portion of hot water. If any difficulty is experienced in removing the precipitate because of its small amount, this is easily overcome by adding a drop or two of the lead acetate solution to the centrifuge tube. The delead liquid is transferred to a Kjeldahl flask, the volume made up to about 150 cc., 2.0 gm. of sodium peroxide are added, and the mixture is boiled down to a volume of 10 to 15 cc. (until it starts to bump). It is cooled, rinsed into a flask, diluted to about

100 cc., and made neutral to litmus by adding sulfuric acid a drop at a time, with constant stirring. Next 10 cc. of saturated silver sulfate solution are added, followed by 1 cc. of normal copper sulfate solution, 0.2 gm. of calcium hydroxide and about 0.5 gm. of magnesium carbonate. The mixture is shaken for 10 minutes. The precipitate should appear grayish; if it does not, more calcium hydroxide is added. The mixture is filtered and the residue washed three times with small portions of hot water. The filtrate is evaporated almost to dryness on the steam bath, 5 cc. of the phenol disulfonic acid reagent, prepared as recommended by Chamot, Pratt, and Redfield (9), are poured in the center of the dish where the nitrates are concentrated and allowed to react several minutes. The mixture is rubbed to a paste with a small pestle, enough water is added to dissolve the salts, and ammonium hydroxide is stirred in until present in excess. Sometimes it is necessary to filter out a slight flocculent precipitate. The solution is made up to some definite volume, usually 50 to 200 cc., and compared with freshly prepared standards as recommended by Bear and Salter (10), using either Nessler tubes or a colorimeter.

SUMMARY.

The various modifications of the Devarda's alloy method for the determination of nitrate nitrogen have been found to be inaccurate in the presence of amide nitrogen.

A modification of the phenol disulfonic acid method has been developed which gives excellent results in the determination of nitrate nitrogen in plant extracts.

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ON A POSSIBLE RELATIONSHIP OF ARACHIDONIC ACID TO THE SATURATED FATTY ACIDS IN FATTY ACID METABOLISM.

BY LAURENCE G. WESSON.

(From the Laboratory of Physiological Chemistry, the Johns Hopkins
University, Baltimore.)

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Hartley (1) in 1909 discovered a tetra unsaturated, twenty carbon, straight chain acid, since named "arachidonic acid," among the acids obtained by the hydrolysis of liver fats. One method which Hartley used to isolate and identify this acid consisted in the addition of bromine to a solution of the mixed saturated and unsaturated acids, and thorough extraction of the precipitated brom acids with benzene. The insoluble residue was found to be octobromoarachidic acid. Later Levene (2) identified one of the unsaturated acids obtained by the hydrolysis of cephalin and lecithin as arachidonic acid. The present writer (3) has shown that when bromine is added directly to an ether extract of brain tissue, a precipitate is obtained, which, after the contaminating groups have been hydrolyzed from the octobromoarachidic acid with hydrochloric acid, gives apparently, in the main, octobromoarachidic acid. The product, because of its very difficult solubility in all solvents, does not lend itself to purification by crystallization. The difficult solubility of octobromoarachidic acid, however, has made possible a procedure based on the last described method of isolating this compound, which is used in the present work for estimating in a comparative way the arachidonic acid content of tissue extracts.

The high degree of unsaturation of arachidonic acid, and the fact that this tetra unsaturated, *twenty* carbon acid is found in animal tissues instead of a tetra unsaturated, *eighteen* carbon acid, are two features that give to arachidonic acid a particular interest. While oleic, linolic, and linolenic acids are dehydrogenated stearic

acids with one, two, and three double bonds in the molecule, no dehydrogenated arachidic acid with one, two, or three double bonds has yet been found in the body. To account for the presence of this twenty carbon, unsaturated acid in the body, Hartley (1) proposed two hypotheses.

1. That it is a desaturation product of arachidic acid. This hypothesis he rejected because of the probable lack of sufficient arachidic acid in the body fats to form as much arachidonic acid as he found in the liver.

2. That this twenty carbon, unsaturated acid is formed *directly* from carbohydrates. In support of this hypothesis, he cited the results of Magnus-Levy (4) and Leathes (5), which indicated the possibility of the synthesis of fatty acids from carbohydrates. Several experiments of his own were also cited. In these he showed that the liver fatty acids from guinea pigs and rats, long maintained on a fat-deficient diet, contained a high percentage of unsaturated acids, formed presumably, he said, from carbohydrates.

The oxidative catabolism of fats in the body takes place in stages which, as far as is known, may be divided into two general types, dehydrogenation and hydroxyl substitution. The first, or dehydrogenation type of reaction, is thought to take place, in part at least, in the liver (6, 1) as a preparative process for the second group of oxidative catabolisms which are brought about in the cells of the various tissues. We have, however, no *direct* evidence that any individual substance is one of what is probably a large group of catabolic products intermediate between the saturated fatty acids (stearic, for example) and the so called "ketone bodies" of the diabetic organism. In the present paper direct evidence is given which indicates that arachidonic acid may be one of these intermediate compounds.

Rats were chosen as experimental animals for the present work for the particular reason among others that the minced whole tissue of a small rat (50 to 150 gm.) is of a weight which is convenient for the method of analysis used. The arachidonic acid (free and combined) contained in the whole animal was estimated by a method which is described in the following section and was found to give values for arachidonic acid which are at least approximately proportional to the amount of that acid in the tissues. By estimating the comparative values on animals that have been subjected to various diets, it was possible to determine whether

or not a given change in the diet affects on the average the amount of arachidonic acid in the animal. The amount of arachidonic acid found in rats per gm. of tissue was, in general, quite constant. For rats in good condition this amount was found to be about 0.75 mg. per gm. of moist tissue. If these well nourished rats were fed over a considerable period of time fats like cod liver oil, which contain arachidonic acid in abundance, the arachidonic acid content rose to 2 or 3 mg. per gm. of tissue. On the other hand, complete deprivation of fats seemed to make but slight difference in the arachidonic acid content of the rats, the average value dropping from 0.75 to about 0.68 mg. per gm. of tissue. Deprivation of all ether-soluble substances, including the fat-soluble vitamin A, gave an average value of 0.65 mg. of arachidonic acid per gm. of tissue. In view of the degree of accuracy of the analytical method, these last mentioned differences are not considered of significance.

Under conditions of fasting, however, the amount of arachidonic acid was found in general to undergo a considerable and significant increase (up to 0.9 to 3.12 mg., averaging about 1.45 mg. per gm. of tissue), during the stages in which body fat is still freely available to the fasting animal but glycogen is not, only to attain more nearly its normal value at the later stages of fasting when the stores of fat are depleted. This increase was also observed in phlorhizinized rats, and in rats which were in the malnourished condition of beri-beri. Since the onset of the increase in the content of arachidonic acid in fasting animals, corresponds somewhat to the partial exhaustion of glycogen stores in the animals, as judged by the length of the fasting period, and in a few test cases by positive reactions for "ketone bodies" in the urine, and since the same phenomenon is observed in phlorhizinized rats, it is suggested, by analogy with the known glucose-fat relationship, that the accumulation of arachidonic acid in the tissues of these rats may be caused by a slowing up under these conditions of an arachidonic acid utilization which is dependent upon the simultaneous oxidation of glucose. Another suggestion is that the increase may be due to the increased demand on the fats for energy under these conditions.

That the arachidonic acid is formed from fats rather than directly from carbohydrates, as suggested by Hartley, by the fasting rat was

also indicated by the increase which was observed after fats were fed at the last period of fasting when the arachidonic acid content has ordinarily become reduced from the abnormal to a nearly normal value.

The observed increases in arachidonic acid were greater than would correspond with the arachidic and higher acid contents of the body and food fats.

Procedure for the Comparative Estimation of Arachidonic Acid.

From 50 to 100 gm. of the macerated tissue are dehydrated in the cold during 24 hours with 95 per cent alcohol, using three portions of sufficient volume to cover the sample.¹ U.S.P. ether is now used for the extraction, during 3 days with six changes of solvent. The combined alcohol and ether extracts are diluted with an equal volume of half saturated sodium chloride solution containing 20 cc. of 10 per cent hydrochloric acid per liter. After thorough extraction of the aqueous layer by ether, the combined ether layers are washed, and evaporated under diminished pressure to a reduced volume. The ether solution is now transferred to a tared flask, and evaporated, also under diminished pressure, to dryness. The residue of ether-soluble material is weighed and dissolved in absolute ether. Bromine vapor, carried by a current of dry carbon dioxide is projected onto the surface of the ice-cold ether solution, until sufficient excess of bromine has collected to color the solution a deep red. The ether solution and precipitated bromoarachidonyl compounds are then allowed to remain for 2 days in a cool place, after which time the precipitate is centrifugalized from the solution in a small tared centrifuge tube, washed with ether and alcohol until the washings are colorless, and allowed to digest overnight with concentrated hydrochloric acid at 40°C. The precipitate is now washed with water, alcohol, and ether, after which it is dried to constant weight at 100°C.

¹ These and the subsequent extractions are conveniently made in a suction filter funnel constructed of a 2 × 4 inch cast iron nipple, and a 2 inch cap through the middle of which a $\frac{1}{2}$ inch hole has been tapped. $\frac{1}{2}$ inch nipples and a $\frac{1}{2}$ inch steam pet-cock complete the funnel. Filtration is through two discs of iron gauze between which a disc of linen is placed. The funnel is covered with an inverted beaker, and the side arm of the filter flask is closed with a cap. The whole is kept in the ice box between filtrations.

The precipitate obtained by this procedure is apparently octobromoarachidic acid, from the weight of which the corresponding amount of arachidonic acid is calculated by the use of the theoretical factor, 0.3225. The "ratio" is the ratio of arachidonic acid to substances soluble in ether.

Test of the Procedure.

Completeness of Extraction of the Ether-Soluble Substances.—The method of cold extraction was tested upon whole rat tissue, minus skin, feet, and tail, since most of the analyses of the present work were made upon this animal. Accordingly, four pairs of duplicate samples of minced rat tissue were extracted according to the above procedure. After the extraction, the ether-soluble substances remaining in the residue were determined by solution of the residue in hot, 20 per cent sodium hydroxide, and subsequent extraction of these from the acidified solution with ether. The residue contained from 2.2 to 4.4 per cent of the total ether-extractable material.

Apparent Completeness of Extraction of the Arachidonic Acid.—The length of time required for apparently complete extraction of the arachidonic acid (free and combined) from rat tissue, is shown by the following experiment. Four equal portions of the minced tissue of a large rat were extracted, one portion for 3 days, one for 4 days, one for 5 days, and one for 6 days. After the 4th day there was no increase in the amount of octobromoarachidic acid obtained, and 4 days was therefore fixed upon as the uniform period of extraction.

The procedure was also tested for apparent completeness of extraction of the arachidonic acid by further extraction of the tissues after the lapse of 4 days with subsequent evaporation of the ether, solution of the residue in absolute ether and bromination in an atmosphere of carbon dioxide. In the two instances in which this was tried no precipitate separated on standing overnight.

Uniformity of Results.—That the procedure gives reasonably constant results was shown by the following experiment. A hog's liver was minced and thoroughly mixed. 75 gm. samples were then weighed out, and extracted for 4 days as outlined above. The results shown in Table I were obtained.

Proportionality of Results upon Samples of Varying Size.—The method was tested for proportional results by analyzing four samples of several tissues, the samples weighing 25, 50, 75, and 100 gm. respectively in the case of the liver, and 30, 40, 50, and 60 gm. in the case of brain tissue. (See Table II.)

Composition of the Precipitate Obtained by the Use of This Procedure upon Whole Rat Tissue.—The washed and dried precipitate, obtained by this procedure from whole rat tissue, was analyzed with the following results.

0.2564 gm. substance: 0.2394 gm. CO_2 and 0.0764 gm. H_2O .

0.1000 " " : 0.1601 " AgBr.

$\text{C}_{20}\text{H}_{32}\text{Br}_2\text{O}_2$. Calculated. C 25.42, H 3.39, Br 67.75.

Found. " 25.47, " 3.33, " 68.14.

TABLE I.

Constancy of Values of Arachidonic Acid and Ether-Soluble Substances by the Method of Analysis.

Sample.	Octobromo- arachidic acid.	Ether extract.	Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
Liver A.					
gm.	gm.	gm.	gm.	gm.	
75	0.2811	4.815	1.21	64	0.0188
75	0.3168	4.966	1.36	66	0.0206
75	0.3195	4.803	1.40	64	0.0219
75	0.2791	4.552	1.20	61	0.0197
Average.....			1.29±0.05*		
Liver B.					
75	0.3618	6.200	1.55	83	0.0188
75	0.3697	6.352	1.61	84	0.0188
75	0.3220	6.116	1.39	81	0.0170
75	0.3319	6.257	1.42	85	0.0170
Average.....			1.49±0.05		

*Average deviation of the mean.

The procedure recommended for the isolation of octobromo-arachidic acid from brain tissue in a preceding paper (3), was used upon whole rat tissue yielding a product which was analyzed with the following results.

0.1614 gm. substance: 0.1512 gm. CO₂ and 0.0476 gm. H₂O.
 0.1000 " " : 0.1591 " AgBr.
 C₂₀H₃₂Br₈O₂. Calculated. C 25.42, H 3.39, Br 67.75.
 Found. " 25.55, " 3.30, " 67.72.

TABLE II.

Approximate Proportionality of Values for the Arachidonic Acid and Ether-Soluble Substances Obtained by the Method of Analysis.

Sample.	Octobromo- arachidic acid.	Ether extract.	Arachidonic acid <i>pro mille</i> .	Ether extract <i>pro mille</i> .	Ratio.
Brain A.					
<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
30	0.2480	2.106	2.14	70	0.0306
40	0.2511	2.520	2.00	63	0.0317
50	0.3442	3.386	2.22	68	0.0328
60	0.3879	4.085	2.09	68	0.0306
Average.....			2.11±0.04		
Liver C.					
25	0.0809	1.800	1.04	72	0.0145
50	0.1934	3.554	1.25	71	0.0176
75	0.3055	5.501	1.31	73	0.0179
100	0.4041	7.546	1.30	75	0.0174
Average.....			1.23±0.05		
Liver D.					
25	0.0923	2.024	1.19	81	0.0147
50	0.1963	4.234	1.27	85	0.0150
75	0.3454	6.326	1.48	88	0.0176
100	0.3999	8.667	1.29	87	0.0149
Average.....			1.31±0.05		

Comparison of the Arachidonic Acid and Ether Extract Contents of "Normal" Rats with Those of Xerophthalmic, Beri-Beri, Fasting, and Phlorhizinized Rats.

Rats Fed a "Normal" Diet.—The group of rats used for the purpose of establishing the normal value for the arachidonic acid content of rats, that is the content on a complete but arachidonic acid-deficient diet, was fed the following diet for 2 months or more

before they were killed: corn-starch, 48; ground rolled oats, 40; technical casein, 5; calcium carbonate, 1.5; sodium chloride, 1; butter fat, 5; iodine in small amounts in the drinking water.

Rats Fed a Fat-Deficient Diet.—These rats were fed for a similar length of time the same diet as were the preceding, except that the unsaponifiable, ether-soluble substances of 5 parts of cod liver oil (Mead and Johnson's) were substituted for the 5 parts of butter fat. (See Table III.)

TABLE III.
"Normal" Rats.

Rat No.	Weight.	Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
Diet included butter fat. "Normal" diet.				
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
6	149	0.69	118	0.0059
7	152	0.76	123	0.0061
8	153	0.81	119	0.0068
9	146	0.75	122	0.0062
Average.....		0.75±0.02		
Diet was fat-deficient.				
10	111	0.68	73	0.0094
11	94	0.64	70	0.0092
12	96	0.71	65	0.011
13	104	0.70	65	0.012
Average.....		0.68±0.01		

Rats Fed a Diet Which Included Cod Liver Oil.—These rats were part of two groups of females that were used in a study of the placental transmission of fats. The rats of both groups were in the last stages of pregnancy, and are comparable with each other. One group was maintained on the "normal" diet, while the other group was fed a diet which included 5 parts of cod liver oil (Mead and Johnson's) in the diet in place of the butter fat. The tendency of the unsaturated fatty acid to be deposited in the adipose tissue along with saturated when fed over a considerable period to well nourished animals is illustrated by these groups (Table IV).

Rats Fed a Diet Which Was Deficient in All Substances Soluble in Ether, Including Vitamin A.—The examination of this group gave an opportunity not only to examine the effect on the arachidonic acid content of rats of the absence from the diet of all substances soluble in ether over a considerable period of time, but also to observe the effect of a condition of severe malnutrition induced by the deficiency of vitamin A in the diet.

The diet upon which these rats were fed was similar to that of the group fed the "normal" diet, except that the butter fat was omitted, and the "technical" casein was alcohol- and ether-extracted. Rats placed upon this diet when their weight was 40 to 50 gm., required 2 or 3 months to attain the eye condition which

TABLE IV.

Pregnant Rats Fed Cod Liver Oil in the Diet Compared with Pregnant Rats Fed the "Normal" Diet.

Rat No.	Weight.	Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
Diet included butter fat. "Normal" diet.				
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
14	188	0.93	83	0.011
15	(174)	(0.31)	(58)	(0.0052)
16	161	0.93	67	0.014
17	93	1.02	98	0.011
18	176	0.73	76	0.0096
Average.....		0.90±0.05		
Diet included cod liver oil.				
19	106	2.17	48	0.041
20	110	2.58	62	0.042
Average.....		2.38±0.14		

results from a deficiency of vitamin A in the diet. The rats when killed were in various stages of eye disease which ranged from a distinct puffiness of one or both eyes to severe ulceration of one or both eyes (Table V).

Rats Which Developed Beri-beri When on the Xerophthalmic Diet.

Some of the rats in the previously described group developed symptoms of beri-beri before the onset of the xerophthalmia.

Diarrhea, dragging of the hind legs, and other motor difficulties were the symptoms noted. (See Table VI.)

Fasting Rats.—Rats which had been maintained for 2 months or more on the "normal" diet, were fasted for varying periods of time up to death (Table VII).

TABLE V.

Rats on a Diet Deficient in All Ether-Soluble Substances. All of These Rats Were Xerophthalmic.

Rat No.	Weight.	Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
21	159	0.73	45	0.016
22	85	0.58	49	0.012
23	76	0.59	51	0.011
24	83	0.45	54	0.0083
25	168	0.71	60	0.012
26	64	0.68	40	0.017
27	68	0.65	11	0.0059
28	79	0.62	33	0.017
29	52	0.74	36	0.020
30	63	0.76	40	0.019
31	58	0.76	35	0.022
32	73	0.55	33	0.017
Average.....		0.65±0.02		

TABLE VI.

Rats in a Condition of Beri-Beri.

Rat No.	Weight.	Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
33	79	1.17	42	0.028
34	63	0.65	20	0.031
35	46	0.68	18	0.040
36	57	1.00	33	0.031
37	51	0.73	17	0.042
38	53	1.66	37	0.045
39	36	1.03	44	0.021
40	52	0.87	34	0.026

Phlorhizinized Rats.—Rats which had been maintained for 2 months or more on the "normal" diet were phlorhizinized by injection of 0.015 gm. of phlorhizin per 100 gm. twice daily for

2 days with fasting, and then killed on the 3rd day. (See Table VIII.)

Livers of Normal, Fasting, and Fat-Fed Rats Examined Separately from the Remaining Tissues.—In order to determine, if possible, whether the major part of the increased amount of arachidonic

TABLE VII.

Rats on a Water Diet.

(Nos. 41 to 49 are arranged in the order of "period of fasting" to correspond with the probable order of decrease of the glycogen stores; Nos. 50 to 64 are arranged in the order of diminishing ether extract factors to correspond roughly with the loss of body stores of fats.)

Rat No.	Weight before fasting.	Weight after fasting.	Loss in weight.	Period of fasting.	Arachi- donic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>days</i>	<i>gm.</i>	<i>gm.</i>	
41	148	138	7	2	0.80	104	0.0078
42	122	113	7	2	0.82	153	0.0054
43	115	105	9	2	0.97	109	0.0090
44	123	103	16	3	0.84	88	0.0096
45	111	82	26	3	0.81	23	0.035
46	135	117	13	4	0.91	85	0.011
47	139	112	20	4	0.84	107	0.0079
48	130	104	20	4	0.76	58	0.013
49	195	162	17	4	1.24	110	0.011
50	145	115	21	5	1.19	111	0.011
51	129	106	18	5	1.08	71	0.015
52	203	165	18	7	1.77	57	0.031
53	185	147	20	7	1.67	47	0.035
54	100	75	25	5	1.13	41	0.028
55	111	79	29	5	1.22	40	0.031
56	199	153	23	7	3.12	39	0.080
57	(99)	(72)	(26)	(5)	(0.82)	(39)	(0.021)
58	115	83	29	6	0.92	30	0.031
59	126	92	27	5	0.79	28	0.028
60	136	86	37	9	0.69	28	0.024
61	143	94	34	7	0.87	24	0.037
62	132	86	35	6	0.75	20	0.038
63	203	119	41	13	0.86	19	0.046
64	104	72	31	7	0.66	15	0.044

acid remained in the liver, or was distributed among the tissues, the livers of a number of animals, normal, fasting, and fat-fed, were examined separately from the rest of the tissues. The

following groups were thus studied: (a) rats fed a "normal" diet; (b) rats kept 4 days on a water diet; (c) rats kept 6 or 7 days on a water diet; and (d) rats kept 6 days on a water diet and then fed fat (lard) for 2 days prior to examination (Table IX).

Distribution of Arachidonic Acid among the Various Tissues.

Eight tissues of the dog were examined for arachidonic acid before the adoption of the analytical procedure in the form given above. The results which were obtained are cited only for the purpose of illustrating the apparently widespread distribution of arachidonic acid in the animal organism (Table X).

TABLE VIII.

Phlorhizinized Rats Compared with Rats Undergoing 2 Days Fasting without Phlorhizin.

Rat No.	Weight before fasting.	Weight after fasting.	Loss of weight.	Arachidonic acid pro mille.	Ether extract pro mille.	Ratio.
Phlorhizinized with 2 days fasting.						
65	gm. (112)	gm. (103)	per cent (8)	gm. (0.83)	gm. (84)	(0.0099)
66	112	107	5	1.07	110	0.0097
67	95	88	7	1.48	155	0.0096
Average.....				1.28±0.14		
Not phlorhizinized with 2 days fasting.						
41	148	138	7	0.80	104	0.0078
42	122	113	7	0.82	153	0.0054
43	115	105	9	0.97	109	0.0090
Average.....				0.86±0.04		

DISCUSSION OF THE TABLES.

An examination of Tables I to IX suggests that apparently two conditions which determine the arachidonic acid contents of rats are:

1. A supply of fats available to the animal from body stores or from food, as illustrated by:

(a). The increase above normal (Nos. 6 to 13) found in general during the stage of fasting when body fat, as judged from the ether extract factor, is still available and freely used by the animal, but glycogen is prob-

TABLE IX.

Livers Examined Separately from the Remaining Tissues.

Rat No.		Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
Livers.				
68-71	"Normal" diet (Group A).	<i>gm.</i> 1.33	<i>gm.</i> 71	0.019
72-74	Fasted 4 days (Group B).	3.55	98	0.036
75-78	" 4 " (" C).	2.63	82	0.032
79-82	" 6 and 7 days (Group D).	1.92	32	0.059
83-85	" 6 days, then fed fat 2 days (Group E).	3.22	106	0.030

Remaining tissues. Rats fed "normal" diet.

Rat No.	Weight before fasting.	Weight after fasting.	Loss in weight.	Period of fasting.	Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>days</i>	<i>gm.</i>	<i>gm.</i>	
68	149	149	0	0	0.63	121	0.0053
69	152	152	0	0	0.72	126	0.0057
70	153	153	0	0	0.78	114	0.0068
71	146	146	0	0	0.72	125	0.0057
Average Group A.....					0.71±0.02		

Rats fasted 4 days.

72	124	104	16	4	0.83	160	0.0052
73	133	110	17	4	0.85	85	0.010
74	134	109	19	4	0.78	109	0.0072
Average Group B.....					0.82±0.02		
75	132	109	17	4	0.84	66	0.013
76	128	102	20	4	0.85	75	0.011
77	112	86	23	4	0.98	108	0.0089
78	94	72	24	4	0.89	66	0.014
Average Group C.....					0.89±0.03		

ably not (Nos. 49 to 56 and 72 to 78), as contrasted with the later stage when body fat has come to a minimum (Nos. 58 to 64 and 79 to 82).

(b). The increase found in the case of rats in the condition of beri-beri

when body fats, but possibly not glycogen, are still freely available to the animal (Nos. 33, 36, 38, and 39), as contrasted with rats in the like condition but whose body fat has become more nearly exhausted (Nos. 34, 35, 37, and 40).

(c). The increase found in the case of rats which were fed fat containing no arachidonic acid, and but little arachidic acid, during 2 days in the last stage of the fasting period (Nos. 83 to 85), as contrasted with the values found for rats that underwent fasting for the same period without this final fat feeding (Nos. 79 to 82).

2. Possibly by a disturbed metabolism of glucose as illustrated by:

(a). The increase found in the case of rats in the "ketosis" period of fasting (Nos. 49 to 56 and 72 to 78) as contrasted with rats on the "normal"

TABLE IX—*Concluded.*

Rat No.	Weight before fasting.	Weight after fasting	Loss in Weight.	Period of fasting.	Arachidonic acid <i>pro mille.</i>	Ether extract <i>pro mille.</i>	Ratio.
Rats fasted 6 and 7 days.							
	gm.	gm.	per cent	days	gm.	gm.	
79	151	111	27	7	0.82	61	0.013
80	179	130	27	7	0.75	31	0.026
81	144	102	30	6	0.66	18	0.036
82	152	102	33	6	0.69	20	0.034
Average Group D.....					0.73±0.03		
Rats fasted 6 days, then fed fat <i>ad libitum</i> , 2 days.							
83	91	68	25	8	0.98	95	0.010
84	106	79	25	8	0.94	113	0.0082
85	124	82	34	8	0.79	44	0.018
Average Group E.....					0.90±0.05		

diet, the fat-deficient diet, and the diet deficient in all ether-soluble substances (Nos. 6 to 9, 10 to 13, 21 to 32, and 68 to 71). If arachidonic acid is formed from carbohydrates, the contrary result would be expected.

(b). The increase found in the case of rats in the beri-beri condition in which a disturbance of glucose metabolism may have been caused by a voluntary fasting induced by a lack of vitamin B, or the absence of a specific functioning of vitamin B (Nos. 33, 36, 38, and 39), as contrasted with rats on the same diet but not in the condition of beri-beri (Nos. 21 to 32).

(c). The increase found in phlorhizinized rats (Nos. 65 to 67), as contrasted with those not under the influence of phlorhizin (Nos. 41 to 43).

On the other hand, the fact that the arachidonic acid factor remains at approximately the normal value (Nos. 6 to 13) in rats when in a condition of severe malnutrition (Nos. 21 to 32), seems to indicate that mere subnormal nutrition or a lowered metabolic rate does not cause the increase in this factor which was observed in fasting, phlorhizinized, and beri-beri rats (Nos. 49 to 56, 65 to 67, and 11 to 32).

If arachidonic acid represents an intermediate stage in the metabolism of the fatty acids, it would appear likely that more fat passes through this stage than would be represented by the

TABLE X.

Data Illustrating the Widespread Distribution of Arachidonic Acid Among the Various Tissues.

Dogs, Nos. 1 to 8.	Arachidonic acid <i>pro mille.</i>
	<i>gm.</i>
Liver.....	1.8
Pancreas.....	1.0
Kidney.....	1.1
Lung.....	0.5
Spleen.....	0.6
Lymph glands.....	0.2
Muscle (heart).....	0.1
“ (temporal).....	0.2

possible arachidic acid content of food and body fats. In the case of the food fats, this is indicated by the comparative constancy of the arachidonic acid factor in rats deprived of fats (Nos. 10 to 13) and of all ether-soluble substances (Nos. 21 to 32) over a considerable period of time (contrast with Nos. 6 to 9). In the case of the body fats, this is indicated by the fact that the arachidonic acid factor increases in 2 days of fasting, from the 3rd to the 5th day, from say 0.84 to 1.16, an increase which, excluding the arachidonic acid catabolized during this period, would require *at the minimum* that about 1.5 per cent of the ether-soluble substances metabolized (about 11 gm. *pro mille* per day) should be arachidic acid. Thus it would appear that a considerable portion of the arachidonic acid found in the rat must have its origin in sources other than arachidic acid. If this is taken into considera-

tion in conjunction with the conclusion that fats, and not carbohydrates as Hartley supposed, are apparently the direct source of at least a considerable portion of the arachidonic acid of the rat, the hypothesis is suggested that in the metabolism of at least an appreciable proportion of the fatty acids, they are built up to the twenty carbon, tetra unsaturated acid, arachidonic acid, apparently in the liver (Nos. 72 to 78 and 83 to 85, as contrasted with Nos. 68 to 71), prior to catabolism in the tissues.

SUMMARY.

1. A method for the comparative estimation of arachidonic acid in the tissues is described.

2. By the use of this method a considerable increase in the arachidonic acid content of rats was detected during periods of active fat but probable subnormal glucose metabolism.

3. The increase was found to be proportionally larger in the liver than in the other tissues of the rat.

4. In rats which were deprived of fats, and, in fact, all ether-soluble substances over a considerable period of time, and in rats which were in a condition of severe malnutrition due to a deficiency in their diet of vitamin A, no considerable change in the arachidonic acid content was noted.

5. Arachidonic acid was found to be present in all dog tissues examined.

6. The hypothesis is suggested that arachidonic acid is an intermediate product in the metabolism of at least part of the fatty acids which contain fewer than 20 carbon atoms.

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SULFUR IN PROTEINS.

II. THE EFFECT OF MILD ALKALINE HYDROLYSIS UPON HAIR.*

By WALTER F. HOFFMAN.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, University of Minnesota, St. Paul.)

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Many investigators have attempted to answer the question as to the forms of sulfur in protein since Fleitmann¹ in 1847 and 1848 showed that the sulfur in proteins may not all be in one compound or be of the same composition. The literature dealing with the effect of alkaline hydrolysis or the effect of alkalies on the sulfur of proteins has been reviewed in an earlier article.²

Human hair is one of the best sources for the preparation of cystine. Before hydrolysis it is desirable to wash the hair free of oils and waxes. This is best accomplished by the use of alkalies. It was found that after human hair had been washed in a *hot* sodium carbonate solution (about 1 to 2 per cent) it was impossible to prepare cystine from the hair by any of the usual methods. The same hair when washed with a *cold* sodium carbonate solution yielded about 5 per cent cystine. A test of the hot sodium carbonate wash solution showed the presence of a sulfide, indicating that sulfur had been removed from the hair during the washing process.

The hair washed with a cold sodium carbonate solution and air-dried contained 4.49 per cent of sulfur when determined by the sodium peroxide fusion method as compared to 3.42 per cent in the hair which had been washed with a hot sodium carbonate solution and air-dried. Thus a loss of about 23.8 per cent of the sulfur from the hair caused a change in the cystine to such an ex-

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¹ Fleitmann, T., *Ann. Chem.*, 1847, lxi, 121; 1848, lxvi, 380.

² Hoffman, W. F., and Gortner, R. A., *J. Am. Chem. Soc.*, 1922, xlv, 341.

TABLE 1.

Analyses for Soluble Sulfur and Nitrogen When 10 Gm. of Hair Were Heated on a Steam Bath with 150 Cc. of 1, 2, and 4 Per Cent Sodium Carbonate for Varying Lengths of Time.

Na ₂ CO ₃	per cent	Time of heating.				
		1 hr.	2 hrs.	4 hrs.	8 hrs.	16 hrs.
1	Mg. of S in filtrate.	26.9	107.1	148.0	168.3	196.6
	Per cent of total S in filtrate.	5.99	23.85	32.96	37.48	43.78
2	Mg. of S in filtrate.	41.2	135.5	170.0	189.6	219.7
	Per cent of total S in filtrate.	9.17	30.18	37.86	42.22	48.93
4	Mg. of S in filtrate.	72.8	146.9	190.0	204.6	250.2
	Per cent of total S in filtrate.	16.21	32.71	42.31	45.56	55.72
1	Mg. of N in filtrate.	12.0	27.4	30.8	54.6	119.4
	Per cent of total N in filtrate.	0.77	1.75	1.97	3.49	7.64
	Mg. of amino N in filtrate.	0.00	4.8	10.7	16.9	44.1
	Per cent of N in filtrate as amino N.		17.52	34.74	30.95	36.93
	" " total N as amino N in filtrate.		0.30	0.68	1.08	2.82
2	Mg. of N in filtrate.	13.9	40.2	60.0	107.0	233.6
	Per cent of N in filtrate.	0.89	2.57	3.84	6.84	14.94
	Mg. of amino N in filtrate.	0.00	11.3	13.1	24.0	54.9
	Per cent of N in filtrate as amino N.	0.00	28.11	21.83	22.43	23.50
	" " total N as amino N in filtrate.	0.00	0.72	1.39	1.54	3.51
4	Mg. of N in filtrate.	15.2	53.6	78.4	98.6	319.0
	Per cent of N in filtrate.	1.03	3.43	5.01	6.31	20.41
	Mg. of amino N in filtrate.	5.6	15.6	26.2	38.2	65.2
	Per cent of N in filtrate as amino N.	24.56	90.10	96.82	98.72	99.44

tent that none could be isolated. There was no appreciable loss in the sulfur of the hair washed in cold sodium carbonate solution.

After observing the above action of dilute sodium carbonate solutions on hair it seemed desirable to study this action in a quantitative manner. 10 gm. samples of air-dry hair were heated in 300 cc. Kjeldahl flasks with air condensers, on a steam bath for 2, 4, 8, and 16 hours with 1, 2, and 4 per cent solutions of sodium carbonate. At the end of the period of hydrolysis the sodium carbonate solution was filtered and the hair washed several times, the wash water being added to the original filtrate. This solution of the filtrate plus washings was made up to volume and analyzed for total nitrogen, amino nitrogen, and total sulfur.³

TABLE II.

Total Sulfur in Hair Which Had Been Heated for 1 Hour on a Steam Bath with Varying Amounts of Sodium Carbonate.

Concentration of sodium carbonate.	Sulfur in hair.	Sulfur in filtrate.*	Total.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
	4.49		4.49
1	4.14	0.27	4.41
2	4.15	0.41	4.56
4	3.77	0.73	4.50

* Percentage of total sulfur in original sample of hair.

The results of the analyses are shown in Table I.

To ascertain if all of the sulfur was accounted for, the total sulfur was determined on the hair which had been heated for 1 hour with 1, 2, and 4 per cent sodium carbonate. The results, Table II, show that all of the sulfur is accounted for.

From the above results it is seen that sulfur is easily removed from hair in comparatively large amounts. The determination of total nitrogen in the filtrate showed that only a small amount of the hair had been hydrolyzed by the most severe treatment. About 25 per cent of the soluble nitrogen is free amino nitrogen. Hair with the sulfur thus removed is identical in appearance to that which has not been treated with hot sodium carbonate.

³ See Hoffman and Gortner² for methods of analyses.

Hair which has been heated with 1 per cent sodium carbonate for even a short time cannot be used as a source of cystine. Even though only about 25 per cent of the total sulfur is removed a change has been brought about, presumably in the cystine molecule, that prevents the formation of cystine crystals during the regular method of cystine preparation.

THE EFFECT OF THE BACTERIAL FLORA ON THE BIOLOGICAL TEST FOR VITAMIN B.

BY V. G. HELLER, C. H. McELROY, AND BERTHA GARLOCK.

*(From the Departments of Chemistry and Bacteriology, Oklahoma Agricultural
and Mechanical College, Stillwater.)*

(Received for publication, May 22, 1925.)

Despite the fact that the presence of vitamins has been recognized for a considerable period of time and that an enormous amount of work has been reported in practically every scientific journal, there has not been found, to date, a single recognized qualitative or quantitative test for their presence. The biological test remains today, as it was in the beginning, the only method of testing for these substances; until the chemist is successful in his endeavors to isolate and analyze the vitamins, we must continue this method, slow and unsatisfactory as it is.

Through the findings of Steenbock, Sell, and Nelson (1) and more recently of Dutcher and Francis (2) considerable discredit has accrued even to the biological tests. These investigators while studying the vitamin B level of rations found that rats reared in the usual laboratory manner grew and developed normally when fed a certain standard synthetic ration, but when placed upon screens so they did not have access to their feces, and given the same ration, they failed to grow. This was shown to be due to a deficiency in vitamin B, for when this substance was added to their ration, they grew and developed normally. This same phenomenon has been observed and reported by Osborne and Mendel (3) in 1911, but they made no attempt to explain it.

Some investigators seem to doubt the importance of this discovery, but there is no doubt that the growth curves in the early stages of feeding are somewhat influenced when the animals are allowed access to their feces. The questions then suggest themselves: First, is the better growth of the rats that have access to

their feces produced by the vitamin which has passed through the bodies of the animals only partially utilized? Second, is the vitamin a catalytic substance that is not destroyed by the process of digestion and is therefore capable of producing its beneficial effects time after time? Third, may it be that the bacteria which grow in the intestinal tract of the animals synthesize vitamin B and store it within their own bodies? The latter theory is worthy of consideration, as it has been demonstrated by numerous investigators that certain microorganisms have the ability to synthesize vitamin B when grown upon media known to be free from this constituent. Damon (4), in his work with the acid-fast bacteria, has contended that three members of this family were capable of synthesizing vitamin B; namely, *Bacillus timothy*, *Bacillus smegmatis*, and *Bacillus moelleri*. The same investigator (5) asserts also that a mucoid organism, Pfeiffer's bacillus, could synthesize and store vitamin B. Portier (6) asserts that mitochondria, which he also calls symbiotes, are merely bacteria adapted to the cell contents and are capable of synthesizing vitamin. Nelson, Fulmer, and Cessna (7) have demonstrated the same for yeast. On the other hand, Damon (8) contended also that *Bacillus coli*, *Bacillus paratyphosus*, and *Bacillus subtilis* did not produce vitamin B. He also claims the same for the spore-forming aerobic organism, *Bacillus adherens*, and the mucoid organism, Friedländer's bacillus.

In an attempt to determine the facts connected with the third theory mentioned above and to study the change in the bacterial flora of the intestinal tract, the following work was undertaken.

EXPERIMENTAL.

Animals weighing approximately 50 gm. were selected from carefully reared stock, for these experiments. In an effort to offset any litter individualities, the rats were divided so that each cage used for comparative purposes contained one animal from the same litter. Six groups were used in the initial experiment. Cages 1, 2, and 3 were round wire cages, 14 inches in diameter, set over tin bottoms of the same size; Cages 4, 5, and 6 were of the same type but were fitted with bottoms of hardware cloth of $\frac{1}{4}$ inch mesh. This false bottom was suspended about 2 inches

above the tin bottom thus permitting the feces to fall through into the tin pan below, so the animals had no access to them.

The basic vitamin-free ration used for all of these determinations was composed of the following:

	<i>per cent</i>
Dextrin.....	76.3
Casein.....	15.0
Butter fat.....	5.0
Salt mixture.....	3.7

The dextrin used in the preparation of these rations was prepared by hydrolyzing starch with 1 per cent citric acid for 3 hours under 30 pounds pressure. The casein was a standard commercial material, which was washed for 5 weeks with acidulated water to remove vitamin B and the salts. The butter was filtered in a hot funnel in the usual way. The salt mixture was McCollum's No. 185 (9).

Two animals, a male and a female, were placed on shavings in Cage 1 and fed the basic ration supplemented by 5 per cent of yeast in place of an equal amount of dextrin. The other cages contained two males and two females each. The animals in Cage 2 were supplied with shavings for bedding; in Cage 3 they were given no shavings, but were placed on pans so they had access to their feces. The animals in Cages 4, 5, and 6 were all placed on the wire screens so they had no access to their feces. All were given the basic vitamin-free ration, those animals in Cage 5 having access also to a cup of shavings placed in their cage as a source of roughage, while those in Cage 6 had the ration supplemented by 5 per cent of agar-agar, to take the place of the 5 per cent of dextrin which had been removed. All animals had an abundant supply of distilled water.

The animals were weighed weekly and the growth curves plotted. Typical curves are shown in Charts 1, 2, and 3. The animals placed in Cage 1, receiving 5 per cent of yeast in addition to the basic ration, grew normally, showing that the ration was complete. The growth curve of animals from Cage 2 is shown in Chart 1. Those in Cage 3 grew less satisfactorily. Chart 2 represents the growth curve of animals fed on screens and Chart 3 those fed on screens but having their ration supplemented with agar-agar (Ration 6).

An inspection of these curves shows undoubtedly that growth ceases and death overtakes the animals much sooner when they

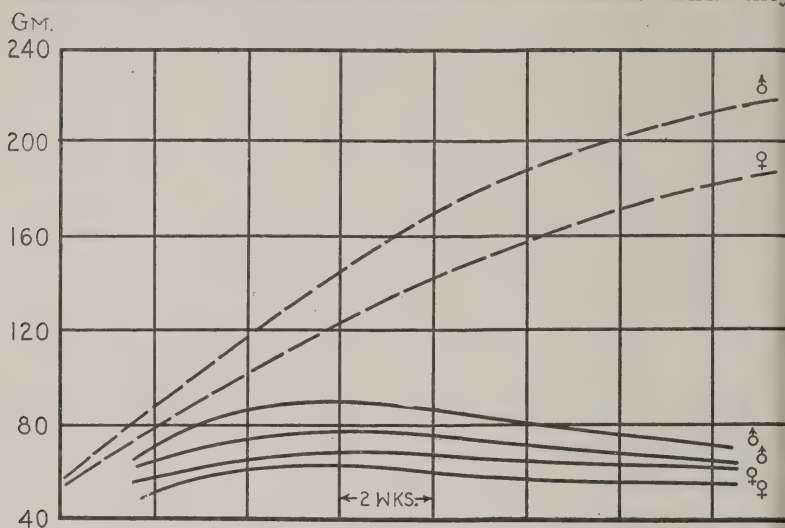


CHART 1.

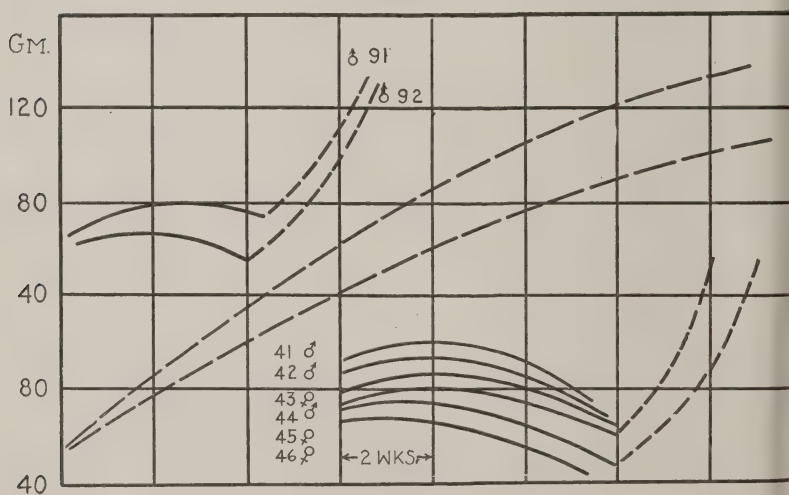


CHART 2.

do not have access to their feces. This is shown in comparing the curve of Animals 42, 44, 45, and 46 in Chart 2 with those of the

animals in Chart 1. The animals not having access to their feces failed more rapidly and died within 7 weeks after the beginning of the experiment, while the animals shown on Chart 1 that had access to their feces lived a much longer period. They grew for practically the same period of time, but their decline was more gradual. When they reached a point a little above their original weight, they seemed to reach a level at which they were able to maintain their weight practically constant for several weeks, showing that in some way they must have been gaining a limited supply of the necessary vitamin during a portion of the time. After

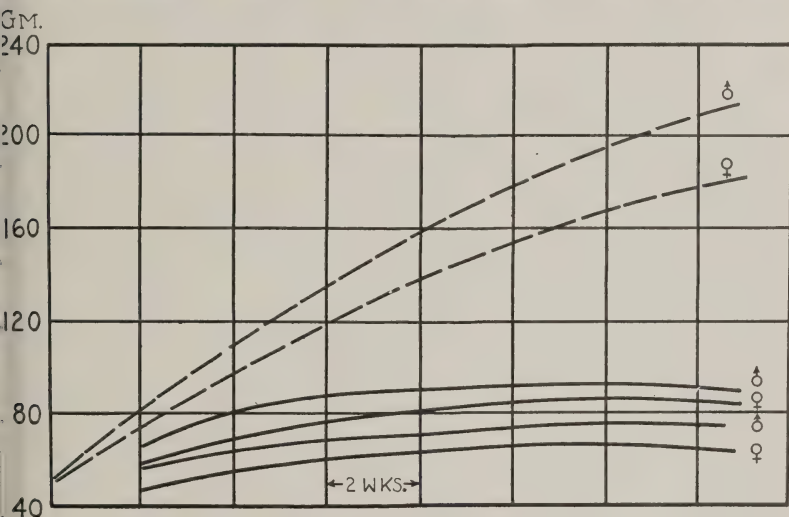


CHART 3.

he animals in Cage 4 died, four more animals were placed in the cage and fed the same ration under the same conditions, with the result that two of them died during the 7th week, at which time the feces from this cage which had been collected and dried to prevent bacterial action, were ground and added to the ration. It was found that when this material made about 20 per cent of their ration, the two remaining animals grew and developed rapidly as shown in the broken line extensions to the curves of Animals 44 and 45, Chart 2. That this growth was due to the addition of the feces was demonstrated by the downward curve of the same animals after the feces were removed from their diet. It is

noticeable then that several factors must be concerned in these curves, as the animals in Cage 5 that had had even shavings as a source of roughage, fared better than those without, while the addition of 5 per cent of agar-agar produced a curve somewhat different from those for the rats in any of the other cages. They not only made a better growth but also failed to decline as rapidly as those that did not have the agar-agar. This shows one of two things: either that other factors are involved besides the benefit from eating the feces, or that the agar-agar contains a trace of vitamin B. Large amounts of the latter substance were extracted with alcohol and the alcoholic extract was added to the basic ration without any appreciable benefit, indicating that the larger bulk in the intestinal tract was beneficial to the animal.

In an effort to determine the effect of the ration on the bacterial flora of the intestinal tract of the rat and to find, if possible, whether or not any organism were present which might act as a synthesizer of vitamin B, the following procedure was carried out: At the close of the 1st week of the experiment feces from the animals in each cage were collected directly from the animals under sterile conditions. This material was weighed and transferred to sterile water blanks, thoroughly macerated, and dilutions of 1:100, 1:1000, and 1:10,000 were made. Plates were poured, using 1 and 0.1 cc. of each dilution. These were incubated for 48 hours. Some of the original dilution from each sample was placed in the fermentation tubes containing lactose broth to determine directly if any members of the intestinal group were present. The formation of gas in each case indicates the presence of this group. After incubating 48 hours the colonies on the plates were studied and counted, the comparative numbers of the various types of organisms per gm. of feces being thus determined. The colonies were then transferred to agar-agar slopes and allowed to incubate 48 hours. At the end of this period the cultures were mounted to determine their morphology. Gram's stain, hanging drop, and sugar media were used as an aid to identification. Each week the samples were collected and studied as described above. The comparative numbers of the organisms in thousands per gm. of the sample are shown in the following tabulation.

Wk.		Cage 2.	Cage 3.	Cage 4.	Cage 6.
1st	Spores.	77	1100	15	19
	Non-spores.	479	22,535	627	767
3rd	Spores.				
	Non-spores.	333	6871	490	456
5th	Spores.				
	Non-spores.	600	10,500	1575	638
7th	Spores.				
	Non-spores.	800	22,000		1360

The figures given are the averages taken from several repetitions of the experiment. Figures for Cages 1 and 5 are not shown, as they had no bearing on the findings. No figures are available for the fourth cage for the 7th week, as the animals either died before that time or the ration was changed.

An attempt has been made to establish a relation between the growth curves of the various lots and the prevalence of the bacteria. Undoubtedly there are several factors involved. The animals in both Cages 2 and 3 had access to their feces, but it has been found that those having access to the shavings as well, do not consume as many of their feces as do those without the shavings. The roughage here seems to be somewhat beneficial, as it was also in the case of those fed on screens with access to shavings. In both Cages 2 and 3, the number of spore-bearing organisms was far in excess of the number found in the cages where the animals were kept on screens. In the same manner the growth curves did not show the drop as found in the case of the animals kept on screens. This is thought to be because the spore-bearing organisms are synthesizers of vitamin B. The prolongation of the growth curve was proportional to the number of the spore-bearing organisms. It is also probable that the effect of the better living conditions of the animals having the roughage in the cage, which was quite noticeable during the colder weather is a factor in these growth curves. McCollum, Simmonds, and Becker (10) believe that comfort is the controlling factor. The animals in

Cage 4 that had no access to the feces showed fewer spore-bearing organisms per gm. of feces than those under other conditions. This would indicate that access to the feces tended to increase the number of spore-bearing organisms in the feces. It is also noteworthy that the number of spore-bearing organisms dropped very rapidly, all having disappeared by the close of the 3rd week. This may be explained through development of autolytic products.

The non-spore-bearing organisms as in the case of the spore formers, are found in greater numbers in Cage 3 where the animals had access to their feces and in much smaller numbers in Cage 4 where the animals were on screens. The organisms in all cases, except in Cage 3, decreased in numbers during the 3rd week. In Cage 3 they had increased greatly. This increase was noticed in all the cages after the 5th week, and was probably due to the greater concentration of the feces, caused by the loss of appetite on the part of the animals on the vitamin-free ration.

From a survey of the literature previously mentioned it was found that the *Bacillus coli* group does not synthesize vitamin B, so it was deemed advisable to study the spore-bearing organisms. An attempt was made to grow these organisms on vitamin-free media, to secure a quantity sufficient to add to the vitamin-free ration, and thus determine if they synthesized and stored vitamin B. Pure cultures of these organisms were obtained by direct transfers. These organisms were inoculated into a cultural media composed of the following.

Beef extract.....	0.3 gm.
Difco peptone.....	0.5 "
NaCl.....	0.1 "
Glycerol.....	4.5 "
Distilled water.....	100.0 cc.

This, Damon (3) in his work with the acid-fast bacteria, demonstrated to be free from vitamin B. After being allowed to incubate for 3 days, the growth was filtered, washed, and dried. 10 per cent of this material was added to the standard vitamin-free ration and fed to the animals which had ceased to grow on the basic ration. The resulting growth was studied, the curves of Animals 91 and 92 being plotted on Chart 2.

From a study of this chart it is seen that when the organisms were added to the ration they produced an increased growth, which is shown in the broken line extensions to the growth curves of Animals 91 and 92. This indicates that vitamin B is synthesized by the spore-bearing organisms that occurred in the intestinal tract of the rat during the early part of the experiment.

These experiments have been repeated until there seems to be little doubt that there is a correlation between the synthesis of vitamin B by the spore-bearing organisms and the extended growth period of the animals. From a close examination of rats fed many types of rations, both on screens and on shavings, it seems that the final results of the biological test are not different under the two conditions. However, the access to shavings and feces retards the final results by several weeks and it would be undesirable to make comparative data between animals fed under the two conditions.

SUMMARY.

1. Experimental animals kept under the usual laboratory conditions do not respond as quickly to vitamin-free rations as do those given the same ration and placed upon screens.
2. Feces fed to animals that have ceased to grow on the experimental ration will produce an accelerated growth.
3. The spore-bearing organisms present in the intestinal tract during the early part of the experiment synthesize and store vitamin B.
4. Animals kept on pans so they have access to their feces have greater numbers of these spore-bearing organisms during the early part of the experiment because of the reingestion of these organisms. The continued growth of these animals is undoubtedly due to the additional vitamin B which has been synthesized and stored by these spore-bearing organisms.
5. Animals given roughage in some form make a better growth than those without this material. This is especially true when agar-agar is incorporated in the ration.
6. Extracts of the various forms of roughage used indicate that the substances contain no vitamin, but the added bulk produces a better physical condition in the animal.

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THE MAINTENANCE OF CARBONIC ACID EQUILIBRIUM
IN THE BODY, WITH ESPECIAL REFERENCE TO THE
INFLUENCE OF RESPIRATION AND KIDNEY
FUNCTION ON CO_2 , H^+ , HCO_3' , AND
 CO_3'' CONCENTRATIONS IN
PLASMA.

BY CECIL D. MURRAY AND A. BAIRD HASTINGS.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

The maintenance of equilibrium by an adult individual, more specifically the maintenance of a steady state, has been recognized as a chief characteristic of life and a cardinal principle in physiology. The principle is one of general application in a qualitative sense, and, if one adheres rigidly to the conditions implied by steady state, *i.e.* excluding transitions from one state to another, the principle is also quantitative. If an individual is maintaining his weight in an environment compatible with life, then it follows, for instance, that carbon excretion is equivalent to carbon intake, that CO_2 elimination is equivalent to CO_2 production in the body, that bone, on the whole, is neither being formed nor dissolved, and that plasma, on the average, must be considered as a saturated solution of the salts of bone. A corollary of the general principle is that the concentrations of various substances must be maintained at levels characteristic for any given state and substance. These are the so called normal levels, and any variations in these are the reflection of mechanisms by which gradients, in a healthy individual, are adjusted to maintain a steady state.

In this paper we propose to discuss the maintenance of steady states with respect to carbonic acid, including the various forms in which it occurs in the body, as CO_2 (or H_2CO_3), as HCO_3' , and as CO_3'' , which is calculated from the ratio $\text{HCO}_3':\text{H}^+$. From the point of view of the principle of maintenance, carbonic acid appears

to be of special interest. On the one hand, free CO_2 , a primary product of widely fluctuating metabolism, is maintained at steep gradients from tissues to lung providing for the continual elimination of large and variable quantities. The percentage change in concentration, from arterial to venous plasma, is greater for free CO_2 than for any other known variable except free oxygen. On the other hand, CO_3'' , the carbonate ion, is associated not only in the immediate equilibrium with the other forms of carbonic acid and water, particularly with HCO_3' and H^+ , but also in the slowly attained equilibrium with Ca^{++} and the solid CaCO_3 of bone. In Section I below it will be shown that the calculated CO_3'' concentration is maintained at a minimum gradient; the absolute change in concentration between venous and arterial plasma is, of course, minute, but even the percentage change is only one-tenth of the percentage change in free CO_2 . Here we will merely point out that a solution which permits wide variations in CO_2 (and to a lesser extent in HCO_3'), but maintains throughout a constant CO_3'' concentration, must have very definite and unique properties. Whole blood plasma under physiological conditions appears to be such a solution and thus serves as a carrier for CO_2 without disturbing the equilibrium with bone in which the CO_3'' ion takes part.

Since theoretically CO_2 , H^+ , HCO_3' , CO_3'' , Ca^{++} , PO_4''' , HPO_4'' , and $\text{H}_2\text{PO}_4'$ are all linked together in any solution in equilibrium with solid $\text{Ca}_3(\text{PO}_4)_2$ and CaCO_3 , it follows that by introducing the CO_3'' concentration in the acid-base chart (the CO_2 , pH, and HCO_3' nomogram for plasma) we can possibly go still further and add the Ca^{++} , PO_4''' , HPO_4'' , and $\text{H}_2\text{PO}_4'$ concentrations. A difficulty exists in that the essential constants for the solution of the problem are not known. Assuming reasonable values and especially that the activity coefficients of the ions in plasma are constant and that a true equilibrium exists, we have prepared a chart which shows the directions and the relative magnitudes of the changes in all the variables for a given change in any one or two of the variables. As in separated blood, the system in equilibrium with CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$ still possesses two degrees of freedom at constant temperature. Using this chart but without necessarily involving the new variables, we have made some deductions concerning respiratory and renal function from the point of

view of physiological fitness. This problem is discussed in Section II.

I. CONCENTRATION OF CO_3'' IN PLASMA.

Constancy of the Calculated Concentration of Carbonate Ion, CO_3'' , in Blood Plasma in the Respiratory Cycle.

By using the data obtained on Bock's blood which are presented in the d'Ocagne nomogram published by Henderson, Bock, Field, and Stoddard, it is possible to calculate the percentage change in CO_3'' concentration of plasma in passing through a respiratory cycle. The results of such a calculation are presented in Table I.

TABLE I.

Difference in CO_3'' Concentration in Arterial and Venous Blood.

Data taken from the nomogram of Bock's blood. pCO_3'' calculated from the equation $\text{pCO}_3'' = \text{pHCO}_3' - \text{pH}^+ + 10.22$.

	Serum.		Cells.	
	Arterial.	Venous.	Arterial.	Venous.
pH.....	7.451	7.421	7.309	7.300
pHCO_3	25.40	27.66	13.02	15.00
pBHCO_3	1.595	1.558	1.885	1.824
pCO_3''	4.364	4.357	4.796	4.744

It may be mentioned here that a CO_3'' concentration line for serum and one for cells may easily be added to the d'Ocagne nomogram of Bock's blood.¹ The scale for serum is practically parallel to the broken lines representing the R.Q., and lies approximately on the line 0.95. The scale for CO_3'' of the cells is roughly parallel to the HbO_2 scale and lies from 5 to 10 mm. to the left of it. In each case the CO_3'' concentrations increase downwards.

The change in pCO_3'' of serum and cells in passing from the arterial to the venous condition is, in the case chosen, 0.007 and 0.052, or 1.6 and 13 per cent, respectively. The percentage change in the carbonate concentration of the serum, 1.6 per cent, is notable for the fact that it is even less than the percentage change in cell hydrogen ion concentration which in this instance was 2.1 per cent.

The properties of a system affording such constancy of CO_3''

¹ Henderson, Bock, Field, and Stoddard (1), p. 432.

concentration may be shown in the following manner. The CO_3' concentration of plasma is defined by the equation

$$(1) \quad \text{pCO}_3'' = \text{pHCO}_3' - \text{pH} + \text{pK}_2^*$$

Differentiating with respect to pH at constant (CO_3'') one obtains

$$(2) \quad \left[\frac{\Delta \text{pHCO}_3'}{\Delta \text{pH}} \right]_{\text{CO}_3''} = 1$$

Over short ranges equation (2) is practically equivalent to

$$(3) \quad \left[\frac{\Delta [\text{HCO}_3']}{\Delta \text{pH}} \right]_{\text{CO}_3''} \times \frac{1}{[\text{HCO}_3'] \times 2.3} = 1$$

Hence we see that the maintenance of a constant carbonate concentration in plasma depends on a definite buffer value of blood, $\frac{\Delta [\text{HCO}_3']}{\Delta \text{pH}}$ (Van Slyke (2)), for any given concentration of plasma bicarbonate. A pure solution of NaHCO_3 , with no buffer value with respect to added CO_2 , will show, in accordance with equation (1), *decreasing* CO_3'' concentrations as the CO_2 tension and consequently the H^+ ion, is increased. On the other hand, a highly buffered solution will exhibit *increasing* CO_3'' concentrations as the CO_2 tension is increased, for the reason that there will be a large increase in HCO_3' in the solution with little change in H^+ , in short an increase in the $\text{HCO}_3':\text{H}^+$ ratio and hence an increase in CO_3'' .

One may utilize equation (2) and the data of Table I to examine the degree to which serum and cells deviate from the condition of absolute constancy of CO_3'' concentration. For serum $\frac{\Delta \text{pHCO}_3'}{\Delta \text{pH}} = 1.2$ and for cells 6.8, instead of the ideal value of 1.

Considering bloods of varying composition in individuals under different conditions, we can state the following propositions: the more highly buffered the blood, the greater will be the increase (or the less the decrease) in the CO_3'' concentration of the plasma as arterial blood changes to venous blood of a given difference in CO_2 tension. High buffer values are associated with high hemoglobin concentrations, and with low respiratory quotients, for here

* $\text{pX} = -\log_{10} X$

we are considering the change, in the respiratory cycle, in pH of serum per unit change in HCO_3' . If there were no associated change in HbO_2 with changing CO_2 tensions the change in pH from arterial to venous blood would be about twice as great as it is ordinarily; the greater the change in HbO_2 for a given change in HCO_3' , the less will be the change in pH and the greater will be the physiological buffer value of the blood. For a given change in HCO_3' , large changes in HbO_2 indicate low respiratory quotients. In the case of Bock's blood the CO_3'' concentrations of arterial and venous plasma are identical when the R. Q. is 0.95. With higher R. Q. the arterial CO_3'' is the higher, with lower R. Q. the venous CO_3'' is the higher.

The general conclusion of the discussion of this section is that in the physiological carbonic acid equilibrium of *plasma* the A-V changes (the arterial-venous changes) are such that the CO_3'' concentration remains more nearly constant than does any other variable involved in this equilibrium. This fact makes it simple to extrapolate determinations on venous blood to theoretical arterial conditions with a fair degree of accuracy, and to draw lines roughly representing A-V changes on any nomogram with a CO_3'' scale or axis. The fitness of the mechanism by which constancy of CO_3'' concentration in plasma is maintained has been indicated and shown to depend on the fact that in blood slight variations in CO_3'' concentration occur with changes in the concentration of buffer substances, and with changes in the respiratory quotient.

II. RESPIRATION AND KIDNEY FUNCTION.

General Deductions from an Acid-Base Chart.

Theoretical Considerations of the Equilibrium Plasma-Bone.

Including the carbonate, CO_3'' , lines in Van Slyke's original acid-base diagram (3) and spacing the variables logarithmically as Peters did (4) (except that he used \log total CO_2 and $\log \text{CO}_2$ tension, instead of pHCO_3' and pH , as the rectangular coordinate background) yields a chart of considerable interest and fundamental symmetry. Fig. 1 is a chart of this character. All the values, pH , pHCO_3' , etc., are expressed in the same unit, the negative logarithm of the molar concentration. Increasing pX values

indicate decreasing concentrations. In Fig. 2 the actual concentrations, except for pH, are used, but otherwise point for point the figures are identical. For the sake of concreteness we have used probable values for certain constants, although many of them are unknown for the conditions prevailing in plasma. That the values designating the several lines are equal each to the logarithm of the *activity*² of the corresponding variable plus a constant must be a strictly thermodynamic fact as long as equilibrium exists in the plasma and as long as the plasma can be considered as a uniform solvent with constant properties over the range of the chart. The activities of ions are usually of greater physiological importance than their total concentrations.

We have made the further assumptions that plasma is in equilibrium with solid CaCO_3 and solid $\text{Ca}_3(\text{PO}_4)_2$, considered as the chief mineral components of bone. For this purpose, in addition to the constants used in Section I above, we have used for this chart the following relations.

$$(4) \text{pCO}_3'' + \text{pCa}^{++} = 7.0$$

$$(5) 3\text{pCa}^{++} + 2\text{pPO}_4''' = 24.1$$

$$(6) \text{pPO}_4''' + \text{pH} = \text{pHPO}_4'' + 12.4$$

$$(7) \text{pHPO}_4'' + \text{pH} = \text{pH}_2\text{PO}_4' + 6.8$$

Having first constructed the nomogram for the carbonic acid equilibrium, the pCa^{++} values are put directly on the pCO_3'' lines to conform with equation (4) and similarly pPO_4''' values are written in on these same lines to satisfy equation (5). The pHPO_4'' lines can be placed by equation (6) and the last equation fixes the $\text{pH}_2\text{PO}_4'$ lines. Curves for total inorganic phosphate ($\text{HPO}_4'' + \text{H}_2\text{PO}_4'$) and for total $\text{CO}_2(\text{H}_2\text{CO}_3 + \text{HCO}_3')$ could be included if desired. In referring to this chart we will speak of a CO_2 line, meaning thereby an iso- CO_2 tension line, any point on which represents the same CO_2 tension. The CO_2 tension axis is considered as a line at right angles to the CO_2 lines. Similarly we will speak of CO_3'' lines, and the CO_3'' axis, and so on.

² Activity is used in the thermodynamic sense introduced by G. N. Lewis to indicate the effective ion concentration. For practical purposes it may be defined as $\alpha = \gamma c$ where α is the activity, c the molar concentration, and γ a factor known as the activity coefficient. For a more complete discussion of the conception of activity Lewis and Randall's "Thermodynamics" (5) may be consulted.

Although solid CaHPO_4 perhaps does not occur in bone, it is interesting to note that if it did, then serum, to be in equilibrium with the three solid salts, would become a uni-variant solution at constant temperature. This follows from the additional requirement that $p\text{Ca}^{++} + p\text{HPO}_4''' = \text{a constant}$, for a uniform solvent, and hence all the variables of the chart would be determined by a single line, and this line would be a CO_2 line, the particular line being determined by the value of the constant. Increasing CO_2

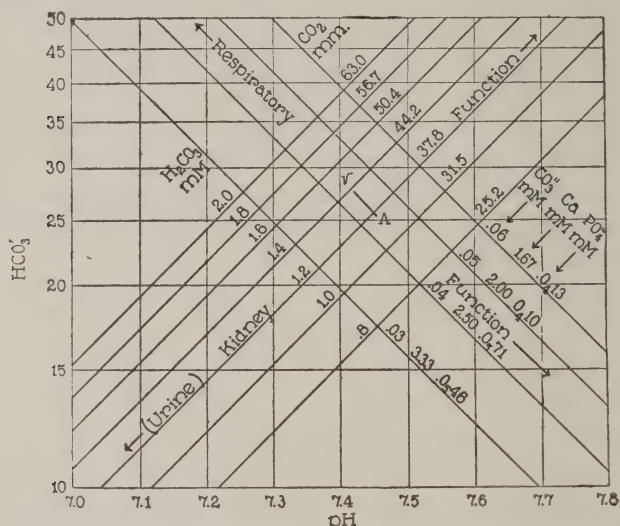


FIG. 2. The chart is similar to Fig. 1 except that the values are expressed in millimols and the lines, except for pH, are drawn accordingly. Areas representing tetany and coma, etc., can be mapped out on this chart (compare Van Slyke (3)). The values under Ca^{++} and PO_4''' are relative values of the activities assuming equilibrium between plasma and bone.

tensions tend to cause precipitation of CaHPO_4 , in a solution in equilibrium with CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$, and decreasing CO_2 tensions tend to dissolve any CaHPO_4 that may be present.

The diagrams (Figs. 1 and 2) represent the maximum that we can at present infer about plasma in the light of any equilibrium between plasma and bone. It is interesting to note that Ca^{++} activities decrease as the conditions of plasma tend to move into the upper right-hand corner of the chart, the tetany area, and increase as conditions approach the lower left-hand corner, the

coma area. At best, however, the equilibrium is probably slowly attained, and we must remember that we are dealing here with an average condition of plasma.

Theoretical Considerations of the Arterial-Venous Cycle and Cardio-respiratory Stimulation.

Confining our attention now to the equilibrium involving carbonic acid in its various forms, we place the arterial and venous points on the nomogram. The data are taken from Table II which was compiled from the nomogram of Bock's blood, published in the article by Henderson and others, and already referred to. In

TABLE II.

Arterial and Venous Concentrations of O_2 , HbO_2 , CO_2 , HCO_3 , pH , CO_3'' , and Cl —Taken from the Nomogram of Bock's Blood.

	Arterial.			Venous.		$\Delta \log$.
			$\log \pm$		$\log \pm$	
Serum.	O_2 mm.	78.0	1.892	34.5	1.538	0.354
	CO_2 mm	1.05	2.021	1.23	2.090	0.069
	(HCO_3') mm	25.40	1.595	27.66	1.558	0.037
	pH		7.451		7.421	0.030
	(CO_3'') mm	0.0432	4.364	0.0439	4.357	0.007
	(Cl) mm	99.36	1.003	98.22	1.008	0.005
Cells.	HbO_2 mm	8.50	2.070	5.77	2.249	0.179
	(HCO_3') mm	13.02	1.885	15.00	1.824	0.061
	(CO_3'') mm	0.016	4.796	0.018	4.744	0.052
	(Cl) mm	50.96	1.293	53.22	1.274	0.019
	pH		7.309		7.300	0.009

the table we have merely included the logarithms of the values for each variable as it exists in arterial and in venous cells and plasma, and in a separate column we have placed the differences, in logarithmic units, between the arterial and venous values of each variable.

We propose now to examine the requirements which must be fulfilled by any mechanism which controls respiratory rate and heart rate, more specifically the effective ventilation rate and the

rate of actual blood flow. For the time being we will assume parallelism between these two rates, and speak of respiration alone. Since we are primarily concerned with carbonic acid, in the discussion which follows we will disregard the rôle played by oxygen. Although oxygen is probably the most important variable in the whole respiratory process, if the oxygen factor, however it may operate, is kept constant, the arguments will be valid. With this restriction, the function of respiration is simply the elimination of CO_2 , the transformation of venous into arterial blood, or what might be described as motion of blood plasma from point V to point A on the nomogram. The actual position of the A-V line (joining arterial and venous points) may be quite different from the one given in Figs. 1 and 2, but A-V lines are always very nearly parallel to the one given. If the buffer value of the blood increases, or if the respiratory quotient decreases, the line becomes a trifle steeper, but the possible variations are not large. The A-V line is practically parallel to a CO_3'' line, which in our chart is also parallel to the CO_2 tension axis.

The principle of the maintenance of a steady state involves the necessary equality of CO_2 production and CO_2 elimination—otherwise the individual would surely die. This biological argument is analogous to a thermodynamic argument: in each case the basis rests upon experience, on the one hand that the organism survives and maintains a steady state or physiological equilibrium, on the other hand that energy is conserved and that equilibrium represents the most probable configuration. In neither case is there any definite suggestion of the mechanism involved, but we can be sure that whatever hypothesis is suggested to explain the mechanism, this hypothesis must be consistent with the more general biological or thermodynamic conclusion. Vicious circles and perpetual motions are barred. In the problem of respiration, stimulation of respiration must operate through a mechanism consistent with the necessity of maintaining equality of CO_2 production and CO_2 elimination. That respiration can maintain any CO_2 tension within limits compatible with life is obvious enough. And respiration being the mechanism for controlling CO_2 tension, it is clear that respiration in the last analysis is controlled by CO_2 tension (barring for the present the rôle of oxygen). Conversely it is inconceivable that a variable which is wholly independent of

and unchanged by the action of respiration should affect the respiratory rate.

The supposition that increased respiration follows from increase in the CO_2 tension of the blood is in harmony with the observation that injections of NaHCO_3 often cause increased breathing. This observation has been considered as an anomaly according to the theory of respiratory control by pH, because the injection of bicarbonate always makes the blood more alkaline and diminished breathing would be expected to follow according to the theory. However, in spite of the increased alkalinity, if the injected solution is more concentrated in respect to bicarbonate than is the blood, there is always an increase in CO_2 tension. This increase is due to the decomposition of the injected bicarbonate solution as it mixes with the blood, the blood being more acid than the injected solution. The CO_2 derived from this decomposition (or the high CO_2 tension of a relatively concentrated NaHCO_3 solution previously adjusted to the pH of blood) is presumably the stimulus to increased breathing. The increased respiration following injections of acid, during exercise, etc., is invariably associated with increase in CO_2 production, whether by decomposition of bicarbonate or by increase in metabolism.

The conclusion arrived at by the biological argument is that any mechanism of respiratory control must be consistent with the maintenance of CO_2 equilibrium. This mechanism must operate largely through changes in the CO_2 tension of the blood, and independently of other variables except oxygen. The most conservative general statement would be that respiration is controlled by the vensity of the blood, by the combination of factors and variables which distinguish venous from arterial blood.

Now let us consider the A-V line. If respiration results in motion from V to A, it is altogether probable that motion from A to V results in respiration, in other words that the stimulus for breathing is derived from any motion of blood plasma which has a component in the A-V direction. It is reasonable to suppose the the most efficient stimulus is one which operates actually along the A-V line, and conversely that a variable which remains constant along the A-V line could not act as stimulus at all. Now the A-V line is practically parallel to the CO_2 axis, and CO_2

may therefore be said to act along the A-V line. The importance of CO_2 tension as a possible respiratory stimulus is not only greater than that of other variables (except oxygen) but it is practically a maximum; that is, the slope of the A-V line is such that a given degree of motion along the A-V line represents practically a maximum change in CO_2 tension with a minimum combined change in pH and pHCO_3' . The slope of the A-V line, as shown in Section I, is due to the specific buffer properties of blood, including the important contribution derived from changes in HbO_2 . Although the buffer properties of blood have been interpreted as part of a mechanism for preservation of neutrality they also insure that CO_2 exchange in lungs and tissues operates at maximum efficiency (providing maximum change in CO_2 tension, minimum combined change in pH and pHCO_3' , and practically no change in pCO_3''). Furthermore the buffer properties of blood insure that respiratory stimulation depends primarily on CO_2 tension (and presumably on oxygen) thereby providing maximum association between the function of CO_2 elimination and the logical stimulus, providing minimum association between respiration and pH or pHCO_3' , and no association with pCO_3'' . The properties of blood exhibit a fitness to maintain a steady state with respect to carbonic acid, allowing maximum changes in CO_2 , and no changes in CO_3'' .

Kidney Function.

Whereas the lungs eliminate large quantities of CO_2 , such small amounts of any form of carbonic acid are found in the urine that the kidney can hardly be said to control the carbonic acid of blood. However, CO_2 (or H_2CO_3), an unionized molecule and freely diffusible, is found in the urine at about the same concentration as in blood, at tensions between that of arterial and venous blood, as shown by Gamble (6). In so far as respiration may maintain a constant average blood CO_2 tension, it follows that the kidney must excrete urine at the same average CO_2 tension, and that the kidney, including NH_3 production as part of the acid-base function, can control the motion of plasma only along a given CO_2 line. The acid-base chart can be used for urine (making small allowances for the change in pK' values for changing salt concentration) and the equilibrium with respect to carbonic acid in urine can be described

as limited to motion along the extended CO_2 line of blood. Since the kidney controls motion of plasma along this line, it is probable that kidney function (operating largely through other variables, phosphates and base especially) maintains a condition in plasma such that the CO_3'' concentration, represented by a line at right angles to the direction of controlled motion, remains relatively constant.

Respiration was conceived as effecting motion along the CO_2 axis, and thereby controlling the CO_2 tension of blood. The kidney, on the other hand, effects motion along the CO_3'' axis, presumably controlling the CO_3'' concentration. Disturbances in either function alone, or unusual strains imposed upon either function, will result in motion of plasma along a line parallel to the line characteristic of each function. A combination of disturbances or strains will result in motion having components parallel to the lines characteristic of each function. The kidneys and the cardiorespiratory system, acting at right angles to each other, are able together to maintain the plasma at a point.

We wish particularly to thank Dr. Van Slyke for his helpful suggestions and criticisms in the development of this paper.

SUMMARY.

The constancy of the carbonate concentration of plasma during an arterial-venous cycle has been shown and the unique fitness of the blood for the maintenance of this constancy has been demonstrated.

A nomogram embodying the variables CO_2 , HCO_3' , H^+ , CO_3'' , Ca^{++} , PO_4''' , HPO_4'' , and HPO_4' has been described. A consideration of the changes occurring in blood during an arterial-venous cycle leads to the hypothesis of respiratory control by CO_2 tension.

Considered from the point of view of the nomogram, respiration causes change of plasma along a line practically parallel to a constant CO_3'' line, whereas kidney function results in change of plasma along a line parallel to a constant CO_2 tension line.

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PREPARATION, SOLUBILITY, AND SPECIFIC ROTATION OF WHEAT GLIADIN.

BY D. B. DILL AND C. L. ALSBERG.

(From the Food Research Institute and the Department of Chemistry, Stanford University, California.)

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INTRODUCTION.

The development of a reliable method for evaluating flour is a matter of great economic importance. The properties of flour depend largely upon the proteins it contains. Though these have been studied often, much still remains to be done. Their physical properties, for example, are far from completely known. The object of this investigation was to contribute information of this kind. A study has been made of the solubility of gliadin in certain solvents and of its specific rotation in certain solvents. Incidentally the method of preparing pure, unaltered gliadin has been investigated and some observations have been made on its denaturation by alcohol.

Preparation of Wheat Gliadin.

The method followed for preparing gliadin was based on the procedures of Osborne and Harris (1906-07), Gróh and Friedl (1914), and Woodman (1922). Certain new features were introduced and preparations of unusual purity and low ash content were obtained. Hence the method adopted will be described in some detail.

Preparation of the gluten, extraction therefrom of the gliadin with dilute alcohol,¹ and concentration of the filtered extract under diminished pressure below 50°C. were carried out essentially as described by Osborne and Harris. The concentrated syrup was then poured into about 5 volumes of 1 per cent aqueous sodium chloride solution contained in a 2500 cc. wide

¹ All the alcohol used was purified by distillation from alkali.

mouth bottle. Vigorous shaking for 1 or 2 minutes precipitated the gliadin as a foam which presents a large surface and is of such a nature that the bottle may be inverted and the wash water drained out. Three vigorous shakings with 1 liter portions of 0.1 per cent sodium chloride solutions followed.

The wet foamy precipitate was dissolved by the addition of a suitable amount of 95 per cent alcohol.² Knowing the volume of 95 per cent alcohol added and the volume of the solution obtained, it was possible to adjust the final solvent to a concentration of approximately 70 per cent of alcohol. This solution was held in the refrigerator at about 5°C. for 24 hours. Much of the gliadin had then separated as a honey-like layer. It appeared to carry down with it most of the lipoids and suspensoid impurities which are difficult, if not impossible, to remove by filtration. The decanted supernatant liquid was turbid, but became water-clear when warmed to room temperature. The honey-like residue was dissolved in warm 70 per cent alcohol and a second separation was carried through in the same manner. A third separation did not succeed.

This method of purifying gliadin is advantageous in that tedious filtration through filter paper pulp is unnecessary. In fact it was found that the gluten extract could be carried through this separation without filtration. Such a separation can only be achieved at the expense of some gliadin and hence is not applicable when a quantitative yield is desired. This method is possibly adaptable to the preparation of other proteins; almost certainly to the preparation of other prolamins.

The clear gliadin solutions from the first and second separations were combined and concentrated under diminished pressure. The gliadin was precipitated and washed in salt solution as described before except that sodium chloride was replaced by lithium chloride. The use of lithium chloride in this and subsequent precipitations renders the removal of electrolytes during the final dehydration with alcohol and ether more nearly attainable.

The wet gliadin was dissolved as before in the minimum quantity of strong alcohol. It was then precipitated by pouring it in a fine stream into 4 volumes of absolute alcohol, containing 0.025 per cent of lithium chloride. Vigorous shaking promoted precipitation. The precipitate was dissolved in about 500 cc. of warm 60 per cent alcohol and precipitated as before. This process was repeated a third time.

The final precipitate was dehydrated twice with absolute alcohol, ground in a mortar, again dehydrated with alcohol, and finally three times with dry aldehyde-free ether. It was then dried at 40°C. and 20 mm. pressure.

Two duplicate preparations, Nos. 3 A and 3 B, were obtained from a Turkey Red wheat patent flour by this method. These preparations formed water-clear solutions in 50, 60, and 70 per

² The percentage of alcohol in this and subsequent cases is percentage by volume.

cent alcohol. No turbidity developed in these solutions after a month at room temperature.

Three other gliadin preparations which did not form clear solutions in dilute alcohol at room temperature had been obtained by a different method. This earlier method differed in an important detail from the method just described. It is necessary, in order to make clear the significance of this detail, to describe some of the conditions under which gliadin may be denatured by alcohol. Osborne (1924) reported that under certain conditions zein may be denatured by alcohol. In the course of the present investigation it became evident that gliadin also may be denatured by alcohol. After standing for some time in contact with 75 to 85 per cent alcohol it becomes irreversibly altered. The conditions are difficult to reproduce for the condition of the gliadin, *i.e.* whether anhydrous or moist, and the temperature are important variables. Under some conditions even 70 per cent alcohol may denature gliadin. If dry gliadin is covered with 70 per cent alcohol, it becomes solvated, forming a concentrated, clear viscous solution below the larger part of the solvent. If this is then allowed to stand quietly for 2 or 3 days, part of the gliadin will be altered and will not dissolve in any concentration of alcohol at room temperature.

In making these three first preparations, an insufficient amount of absolute alcohol was used for precipitation. The gliadin came down as a viscous translucent mass rather than in the form of opaque white flakes. This translucent mass dissolved only slowly in 70 per cent alcohol. Here is where denaturing occurred. The difficulty may be avoided by using a large excess of absolute alcohol and by redissolving in 60 per cent rather than in 70 per cent alcohol. The use of 60 per cent alcohol is advisable because the strong alcohol held by the precipitate raises the concentration of alcohol in the solvent.

In spite of the fact that these earlier preparations did not dissolve completely in any concentration of alcohol at room temperature, each contained a considerable proportion of unaltered gliadin. This unaltered portion was readily separated. Each preparation was dissolved in about twenty times its weight of warm 50 per cent alcohol. Enough strong alcohol was added to make a solvent containing 70 per cent alcohol by volume. This was allowed to stand overnight in the refrigerator to allow the denatured

gliadin to settle out, and from this point on was carried through the remainder of the method first described above. Each of these first three preparations yielded about one-half its weight of apparently unaltered gliadin.

The preparations finally obtained were five in number: one from a commercial family patent flour, two duplicate preparations from a Kansas Kharkoff flour, and two duplicate preparations from a

TABLE I.
Nitrogen and Ash Contents of Five Gliadin Preparations (Dry Basis).

Preparation No.	Nitrogen.		Ash.
	Individual determination.	Average.	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	17.55	17.55	0.06
2 A	17.49 17.39 17.41	17.43	0.11
2 B	17.56 17.50 17.53	17.53	0.07
3 A	17.45 17.73 17.74 17.73	17.66	0.07
3 B	17.46 17.51 17.58	17.52	0.08
Average.....		17.54	0.08

Turkey Red flour. These will henceforth be referred to as Preparations 1, 2 A, 2 B, 3 A, and 3 B.

The moisture content of these preparations was accurately determined by heating 1 gm. portions to constant weight at 115–118°C. and atmospheric pressure. (Cf. Benedict and Osborne, 1907.) All subsequent determinations have been calculated to the dry basis.

The nitrogen and ash contents of these preparations are shown in Table I. Nitrogen was determined by the Kjeldahl-Gunning method, using K_2SO_4 and $CuSO_4$. Correction was made for the blank and the precautions advised by Paul and Berry (1921) were observed. Digestion was carried on for 3 hours after clearing. Ashing was performed in a platinum dish at low red heat.

The values reported in the literature for the nitrogen content of wheat gliadin have been collected in Table II. Osborne and Voorhees (1893) were particularly concerned with obtaining quantitative separation of each of the proteins. Most other investigators have concerned themselves with the question of isolating pure gliadin for study of its physicochemical properties. The evidence

TABLE II.
Nitrogen Content of Wheat Gliadin.

Observer.	No. of samples.	Nitrogen (dry basis).
		<i>per cent</i>
Osborne and Voorhees (1893)	27	17.66
Mathewson (1906, <i>a</i> ; 1906, <i>b</i>)	2*	17.54
Gr6h and Friedl (1914)	2	17.53
L6uers and Ostwald (1920)	1	17.2
Cross and Swain (1924)	4	17.53
Eto (1924)	1	17.2
Tague (1925)	1	17.51
Dill	5	17.54

* Mathewson was not certain that one of his preparations was anhydrous.

suggests that the generally accepted nitrogen content of gliadin which was established by Osborne and Voorhees is slightly high.

Each of the five preparations made in the course of this investigation was quite pure. The ash content may be regarded as negligible (*cf.* Loeb, 1922). One preparation was examined for lipoids by Hertwig's method (1923) and found to contain less than 0.02 per cent. The percentage of ammonia nitrogen formed on acid hydrolysis was determined by the method of Van Slyke (1911-12). Two determinations on one of these preparations gave 26.10 and 26.23 per cent as compared with 25.57 per cent found by Van Slyke (1911-12); 26.01 per cent found by Blish (1916); and 26.40 per cent found by Cross and Swain (1924). Each of these last two values is the average of a number of determinations.

The altered gliadin, separated from the earlier preparations, has been examined. These first preparations could be dissolved in warm 70 per cent alcohol. However, during separation and drying with absolute alcohol and dry ether, further alteration took place for the dried material could not be completely dissolved in any concentration of alcohol at any temperature. On the dry basis it contained 17.14 per cent of nitrogen, and after acid hydrolysis 25.85 per cent of the total nitrogen was found to be ammonia nitrogen. When placed in warm 70 per cent alcohol it became mucin-like. These observations, considered in connection with the fact that this substance had once been soluble in 70 per cent alcohol at room temperature, leave no doubt that this material is altered gliadin. It is probably identical with the "residue" analyzed by Cross and Swain (1924) and possibly identical with the mucedin or with the gluten-fibrin of Ritthausen (1872). This alteration of gliadin is probably quite significant in connection with its determination in a flour. Heretofore it has been tacitly assumed that gliadin may be treated with any concentration of alcohol with impunity. Further, the preparation of pure glutenin involves the removal of gliadin with alcohol. If any gliadin becomes denatured in the process, it will remain in the flour or gluten. But the altered gliadin is readily soluble in dilute alkali and it would therefore contaminate the glutenin when alkali is used for its extraction.

Solubility of Wheat Gliadin.

Gliadin and a few other plant proteins exhibit such a unique behavior toward the solvent alcohol-water that they have been grouped together as the prolamins. While insoluble in either pure solvent, they are soluble in suitable mixtures of the two.

That much discussed question, the value of the gliadin-glutenin ratio, will remain unsettled until exact methods are at hand for determining these two proteins. Many methods have been devised for determining gliadin. None of these investigations has been preceded by a careful study of the solubility of pure gliadin in ethyl alcohol-water, although this binary solvent is generally used in the process of determining gliadin. The importance of obtaining quantitative knowledge regarding the solubility of gliadin is clearly evident.

Many studies, such as that of Hoagland (1911), have been carried out in which the amount of nitrogen extracted from flour with alcohol-water mixtures has been determined. But there are many nitrogen-containing compounds in flour and hence nothing conclusive regarding the solubility of gliadin is learned from such an investigation.

Two investigators have made quantitative studies of the solubility of pure gliadin. Greaves (1911) states: "The solubility in 70 per cent alcohol of carefully prepared gliadin was found to be 0.0601 while in 74 per cent alcohol it was found to be 0.0538." Eto (1924) concludes that it is most soluble in 60 to 70 per cent alcohol and that its solubility is practically unlimited in these solvents above 30°C.

The phenomenon of a substance being most readily dissolved by a binary solvent is not common. Apparently, cellulose nitrate in ether and alcohol, as well as cinchonine in chloroform and alcohol are cases analogous to the behavior of gliadin in alcohol and water. Casein in pyridine and water, studied by Levites (1911), probably is not strictly analogous, for the basic character of pyridine may result in the formation of a caseinate. The finding of Larguier des Bancelles (1908) that gelatin is dissolved more readily by aqueous alcohol or aqueous acetone than by water alone suggests a partial analogy to the behavior of gliadin. In a recent study of the peptization of gelatin by mixed liquids, Mardles (1924) concludes that "the solubility of gelatin is greater in the mixed liquids than the average value, and under some conditions the solvent power of water can be increased, or not appreciably diminished by the addition of a non-solvent such as pyridine, glycerol, alcohol, etc."

The solubility of zein, the prolamin of maize, has been investigated by Galeotti and Giampalmo (1908). These investigators applied the usual method of determining the solubility of crystalloids. Zein was agitated with water-alcohol solvents at 25°C. for 24 hours. The concentrations of the resulting solutions were then determined. About 20 per cent solutions were obtained in 60 and in 70 per cent alcohol. Much less zein dissolved when there was more than 70 per cent or less than 60 per cent of alcohol in the solvent. They did not demonstrate that saturation was reached under the conditions of their experiments. This precaution should not be neglected when one is working with substances which dissolve slowly and which form viscous solutions.

There is a question whether one can refer properly to the "solubility" of a protein. Peptization is a broader and possibly a safer term. Bancroft (1921) states: "If the amount of substance peptized by a given mass of water under given conditions is fairly constant, it will look as though we were dealing with true solubility." With this interpretation it can be said that globulin forms

true solutions, for Osborne and Harris (1905) note that concentrated solutions of globulin prepared above 30°C. "when cooled deposit the globulin either in crystals or in well-formed spherules or spheroids." As pointed out by Cohn (1921-22): "In the case of a protein that is relatively insoluble at its isoelectric point the undissociated protein HPOH may be assumed to have a definite solubility." Zein also would seem to form true solutions in alcohol-water, if one accepts the solubility determinations of Galeotti and Giampalmo (1908).

The uncertainty of securing saturation of a solvent with gliadin by agitation at a fixed temperature led to the adoption of the plethostatic method (so called by Hill, 1923) of determining solubility. Gliadin and solvent were sealed in glass tubes about 0.6×10 cm. Solution was effected by heating the sealed tubes in a thermostat adjusted to 50°C. In this way a series of solutions was obtained in solvents containing 50, 60, and 70 per cent alcohol. The gliadin concentrations ranged from 0.1 to 25 gm. per 100 gm. of solution. It was planned to cool these solution tubes very slowly and note the temperatures at which precipitation of gliadin took place.

The tubes, containing water-clear gliadin solutions, were placed in a water bath at 50°C. The bath was cooled at the rate of 3 to 4 degrees per hour and at the same time was stirred to maintain a uniform temperature. When room temperature was nearly reached it was surrounded by a second bath which was kept at a somewhat lower temperature with ice. Salt was added when the freezing point was approached. The temperatures at which turbidity developed are shown in Table III.

It is evident that for solutions of gliadin in 50, 60, and 70 per cent alcohol, there is, for each solvent, a fairly well defined temperature at which turbidity develops. In solutions of gliadin ranging from 2.5 to 25 per cent this temperature is practically independent of the gliadin concentration. Even below 2.5 per cent concentration, turbidity develops within a few degrees of the critical temperature characteristic of more concentrated solutions.

The question arises, does supersaturation play a rôle in this phenomenon? It was definitely eliminated by warming tubes which had just become turbid. Within 1 or 2 degrees, or in some cases within a few tenths of a degree, of the temperature at which

turbidity had developed, the solutions became clear, regardless of gliadin concentration. In other words, gliadin solutions ranging in concentration from 2.5 per cent up to at least 25 per cent become (for a given solvent) turbid at the same temperature and redissolve at practically the same temperature. This phenomenon appears to be one of peptization rather than of true solution. It appears that within this range of gliadin concentration there is a critical peptization temperature for gliadin in each of these three ethyl alcohol-water mixtures. It is true that for solutions more dilute than 2.5 per cent, the turbidity temperature becomes

TABLE III.

Turbidity Temperatures of Solutions of Gliadin in Alcohol-Water.

Gliadin. gm. per 100 gm. of solution (approximate)	EtOH by volume in the mixed solvent.		
	50 per cent.	60 per cent.	70 per cent.
	Turbidity temperature. °C.	Turbidity temperature. °C.	Turbidity temperature. °C.
0.10	-3	-2	-2
0.20		-1	0
0.40	+5	-1	+3
0.60		0	0
0.80	+6	-1	
1.00		+1	+3
2.50	+7	+2	+6
5.00	+7	+2	+5
10.00		+1	+8
25.00	+8	+2	+8

lower as the gliadin concentration decreases. The behavior of gliadin within this range is similar to that of crystalloids. No further investigation of this region of dilute solutions has been made.

This investigation was then extended to a wider range of alcohol-water mixtures. A series of solutions was prepared, each solution containing approximately 2.5 per cent of gliadin by weight. Each of the five gliadin preparations was used. With one gliadin preparation, in addition to ethyl alcohol-water, varying proportions of water with methyl alcohol and with *n*-propyl alcohol were used. The methyl alcohol used was Baker's absolute.

The *n*-propyl alcohol had a specific gravity of 0.8091 and a boiling point of 97°C. These solutions were prepared as before except that in a few cases the gliadin could not be peptized at 50°C. These were heated to 70°C. for a short time. The data obtained are tabulated in Table IV and shown graphically in Figs. 1 and 2.

Fig. 1 illustrates the behavior of the five gliadin preparations in

TABLE IV.
Turbidity Temperatures of 2.5 Per Cent Gliadin Solutions in Alcohol-Water Solvents.

Alcohol by volume.	Temperature at which turbidity developed.						
	Gliadin 1 in EtOH -H ₂ O.	Gliadin 2 A in EtOH -H ₂ O.	Gliadin 2 B in EtOH -H ₂ O.	Gliadin 3 A in EtOH -H ₂ O.	Gliadin 3 B in EtOH -H ₂ O.	Gliadin 3 A in MeOH -H ₂ O.	Gliadin 3 A in <i>n</i> -PrOH -H ₂ O.
<i>per cent</i>	°C.	°C.	°C.	°C.	°C.	°C.	°C.
2.5				Above 70.			
5.0				66		Above 70.	Above 70.
7.5				56		70	
10.0				44		58	28
20.0				33		43	17
30.0	33	29	32	24	27	34	6
35.0				21			
40.0	23	19	21	16	18	26	-3
45.0				11			-5.5
50.0	13	9.5	12	7.5	9	21	-6.5
55.0	11	7.0	10	4	6.5		-6
60.0	8.5	4.5	7.5	2	5.5	13	-3
65.0	10	5.5	9.0	2.5	6	10.5	
70.0	15	9.5	13	5	11	12	14
75.0				13			
80.0	41	35	38	31	35.5	19	61
82.5				41			Above 70.
85.0				55		29	
90.0				Above 70.		Above 70.	

ethyl alcohol-water mixtures. The curves in Fig. 1 are similar in character but differ in their distance from the *x*-axis. Preparations 2 A and 2 B, although from the same flour, do not exhibit an identical peptization behavior; nor do duplicate preparations, Nos. 3 A and 3 B. This somewhat disconcerting result is subject to three possible explanations: Variable proportions of two gliadins may have been present, as suggested by the work of Lindet

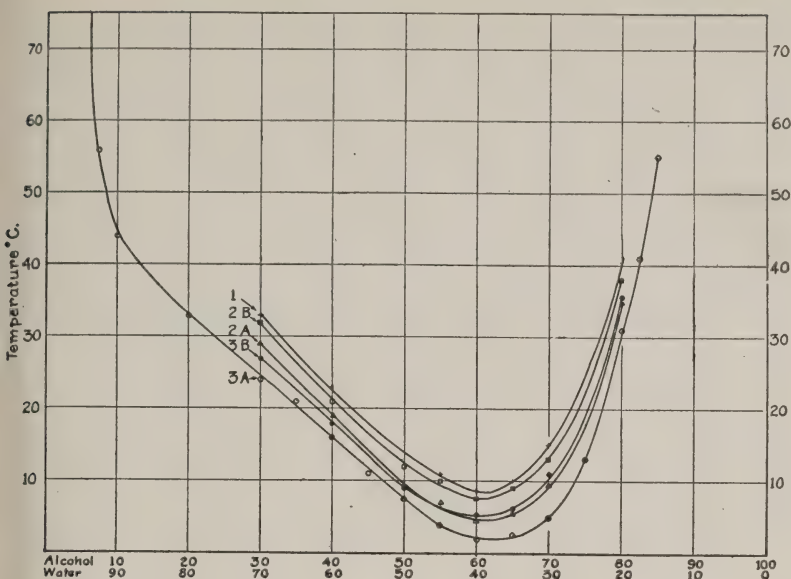


FIG. 1. Critical peptization temperatures of five gliadin preparations in EtOH-H₂O.

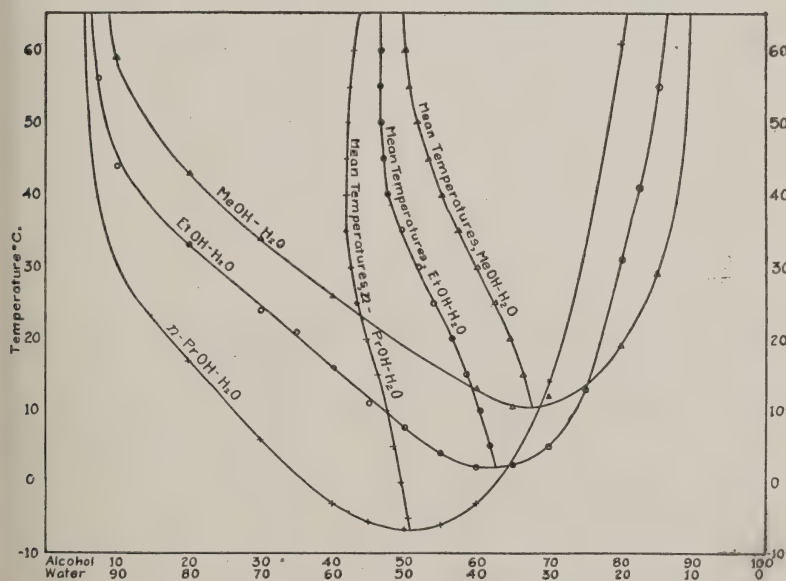


FIG. 2. Critical peptization temperatures of gliadin in MeOH-H₂O, EtOH-H₂O, and *n*-PrOH-H₂O.

and Ammann (1907); variable proportions of impurities may have been present; or variable degrees of denaturing may have taken place in precipitating and dehydrating the gliadin preparations.

The first possibility is remote. Essentially the same methods of extraction were used on the duplicate preparations and a clean-cut or even a considerable separation is improbable. With mixtures of two gliadins the critical solution temperatures in all probability would be those of the gliadin having the higher critical solution temperatures. To test this question, 2.5 per cent solutions in 80 per cent ethyl alcohol were prepared in one of which equal weights of Preparations 2 B and 3 A were used and in the other, 1 part of No. 2 B and 3 parts of No. 3 A. The critical peptization temperature of the first mixture was 38°C. and of the second, 37°C., as compared with 38°C. for a solution of No. 2 B alone and 31°C. for No. 3 A alone. Separation of two hypothetical gliadins is therefore eliminated as an explanation of the question at issue.

The possibility that variable proportions of impurities may be responsible for the difference in critical solution temperatures of duplicate preparations seems unlikely. The preparations were practically lipid-, carbohydrate-, and ash-free.

The possibility of partial denaturing having taken place is worth considering. It will be recalled that the first gliadin preparations were not homogeneous but contained a protein insoluble at room temperature in any concentration of alcohol. This altered gliadin, before separation from the unaltered proportion, could be dissolved in warm 70 per cent alcohol. During the process of separation, however, it became insoluble in alcohol under any conditions. It has been found that heating a solution of gliadin in 80 per cent alcohol at 60°C. for several hours raises the critical peptization temperature several degrees. It is evident that denaturing by alcohol raises the critical peptization temperature curve. If the further assumption is made that the upward shift may take place in imperceptible or slight gradations, a plausible explanation is supplied for the different locations of the critical peptization temperature curve of each of these five preparations. This question should be investigated further, for it has many interesting implications.

However, at present there is no final basis to decide whether it is the presence of impurities or slight differences in degree of dena-

turing which is responsible for the different levels of these parallel curves.

The curves of Fig. 1 reveal a wider range of alcohol concentration capable of peptizing gliadin than has hitherto been realized. Preparation 3 A, which has the lowest critical peptization temperature curve, is peptized at 20°C. in solvents containing approximately 35 to 77 per cent of alcohol. It is peptized in 80 per cent alcohol at 31°C. and even in 10 per cent alcohol at about 44°C. Incidentally, a 20 per cent solution of gliadin was prepared without difficulty in 10 per cent alcohol at 50°C. In fact, gliadin seems to be peptized as rapidly in 10 per cent alcohol at 50°C. as it does in 70 per cent alcohol at 20°C. While no quantitative measurements were made, it appeared that the rate of peptization was roughly proportional to the number of degrees between the critical peptization temperature and the temperature at which peptization was effected. Peptization at any temperature seemed to be most rapid in 60 per cent alcohol.

The solubility of gliadin in 80 per cent alcohol is a moot question. Mathewson (1906, *a*) was unable to dissolve gliadin in 80 per cent alcohol at 20°C. but succeeded in dissolving it in this solvent at 40°C. In agreement with this finding are the curves of Fig. 1; none of these gliadin preparations is peptized in 80 per cent alcohol at 20°C. and all but one are peptized at 40°C. Yet Osborne and Harris (1906-07) determined the specific rotation of gliadin at 20°C. in this solvent.

The curve of Preparation 3 A in Fig. 1 is shown in most detail. It appears to be asymptotic at 5 and at 87.5 per cent alcohol. However, the points determined in alcohol more concentrated than 70 per cent are capable of less accurate determination than the others. As mentioned before, gliadin may be denatured by such solvents. For this reason solutions of gliadin in alcohol more concentrated than 70 per cent were prepared as rapidly as possible and their critical peptization temperatures were determined without delay.

In Fig. 2 are shown the critical peptization temperature curves for Preparation 3 A in methyl alcohol-water, in ethyl alcohol-water, and in *n*-propyl alcohol-water. In each of these mixed solvents there are, for a given temperature, two points on the critical peptization temperature curve (except at the apex). One

point corresponds to the minimum percentage of alcohol which will peptize gliadin at that temperature. The other corresponds to the maximum percentage of alcohol which the solvent can have. At this given temperature gliadin cannot be peptized outside this range of alcohol concentration. The minimum and maximum values at 5 degree intervals have been averaged and these mean values plotted for each of the curves of Fig. 2. The lower portion of this curve is nearly a straight line. Its extension by extrapolation to the critical peptization temperature curve determines the apex of the curve; that is, the percentage of alcohol in the solvent which had the lowest critical peptization temperature.

The lowest point on the methyl alcohol-water curve thus determined is at 10°C. and there is 68.5 per cent of methyl alcohol in the solvent. The corresponding values on the ethyl alcohol-water curve are 2°C. and 63 per cent; on the *n*-propyl alcohol-water curve, -6.5°C. and 51 per cent. These percentages correspond to the following molar concentrations: 17.3, 10.8, and 6.8, respectively.

The ethyl alcohol-water mixtures which peptize gliadin at room temperature cover a much wider range than those of methyl alcohol and water. The range is greatest with *n*-propyl alcohol and water. This gliadin preparation is peptized at 20°C. in a solvent containing from 17 to 72 per cent of *n*-propyl alcohol.

An attempt was made to prepare solutions more concentrated than 25 per cent. No difficulty was experienced in preparing 50 per cent solutions at 50°C. in 50, and in 60, per cent ethyl alcohol. As these were cooled they became extremely viscous and, before the critical temperature was reached, somewhat opalescent. The characteristic turbid appearance did not develop at the critical temperature, due possibly to the extreme viscosity of the solutions. Solutions of about 60 per cent concentration in 50 and 60 per cent alcohol were prepared by holding the sealed tubes at 50°C. for several days. However, these solutions were too viscous for the escape of entrapped air bubbles and hence no attempt was made to reach higher concentrations by this method.

When a solution of gliadin in ethyl alcohol-water was evaporated at room temperature with the occasional addition of strong alcohol, a transparent film was eventually obtained. This film as it approached dryness was under much strain and finally, with a

crackling sound, broke into small pieces. At the same time, pieces of glass were chipped from the containing crystallizing dish, so powerful are the adhesive properties of this gliadin film. A solution of gliadin in 70 per cent *n*-propyl alcohol may be evaporated to dryness at 50°C., the addition of alcohol to prevent turbidity developing being unnecessary. Due to its possessing a vapor pressure near that of water at this temperature, enough *n*-propyl alcohol remains in the solvent to maintain an apparently homogeneous system throughout the entire evaporation. This formation by gliadin of a transparent film is an old observation; it was responsible for the name "plant gelatin" proposed by Liebig.

Evidently there is no upper limit to the solubility of gliadin in suitable alcohol-water mixtures above the critical solution temperature curve. The fact that no upper limit can be established suggests that we have here a system of three liquids. Two of them, water and alcohol, are miscible in all proportions. The third, solvated gliadin, is completely miscible with the other two above the critical peptization temperatures. Apparently both water and alcohol are adsorbed since gliadin is not dissolved, or peptized by either pure solvent.

Ostwald and Wolski (1921) found that zein in 97 per cent alcohol diffused through parchment, collodion, and rubber membranes and concluded that zein was in part molecularly dispersed in alcohol of this concentration. Hoping to learn something of the degree of dispersion of gliadin in alcohol-water, dilute gliadin solutions in 60 per cent alcohol were dialyzed for 8 days against 60 per cent alcohol. Bags of vegetable parchment and of collodion were used. At the end of this time the entire dialysate (about 200 cc.) from each bag was evaporated to small volume and tested for gliadin by the biuret test. The result was negative for the parchment bag and weakly positive for the collodion bag. We can conclude from this experiment that gliadin in 60 per cent alcohol is not readily diffusible through these membranes. Whether the ultimate units are individual protein molecules with adsorbed solvent or whether each unit contains aggregated protein molecules cannot be stated on the basis of this experiment.

The peptization of gliadin by a number of other solvents was investigated. Aqueous solutions of known concentration of a

number of substances were prepared. A measured volume of such a solution was introduced into a 100 cc. glass-stoppered graduated cylinder and enough gliadin was added to make a 1 per cent solution. When the gliadin was completely peptized, the temperature was adjusted to 20° and boiled distilled water was added in small portions and with stirring until turbidity appeared. The volume was noted and the molar concentration of the added substance was calculated. The results are shown in Table V.

The parallel behavior of potassium thiocyanate and ammonium thiocyanate (shown in Table V) indicates that the anion is the ion concerned in this phenomenon.

TABLE V.

Minimum Concentration of Certain Substances in Water Capable of Holding Gliadin in Solution at 20°C. as Determined by Dilution.

Substance.	Molar concentration.
Potassium thiocyanate.....	1.06
Ammonium ".....	1.09
Potassium iodide.....	5*
Urea.....	2.03
Methyl alcohol.....	12.1
Ethyl ".....	5.8
n-Propyl alcohol.....	2.28
Isopropyl ".....	4.17
Chloral hydrate.....	3.0

* As this solution was diluted the foaming tendency was so great that it was impossible to determine exactly the concentration at which turbidity appeared.

Gliadin concentration does not seem to be a limiting factor in the capacity of these solutions to peptize gliadin. 1 and 5 per cent solutions of gliadin were prepared in urea-water. Upon dilution of these two solutions turbidity developed at the same point. 1 and 5 per cent solutions of gliadin in potassium thiocyanate-water behave in the same way upon dilution. The suggestion made by Spiro (1900) that the protein-dissolving power of urea-water may be explained by the formation of an alkali proteinate is rendered unlikely by the above observations. It seems likely that the ability of urea-water to peptize gliadin is closely related, if not entirely analogous, to the ability of alcohol-water to peptize it.

Some additional observations were made on the peptization of gliadin. Glycerol, ethylene glycol, each of the propylene glycols, mixtures of water with each of these, mixtures of glycerol and absolute alcohol containing in excess of 25 per cent glycerol, and mixtures of water with methylethyl ketone containing from 10 to 25 per cent by volume of the latter gave clear solutions of gliadin at room temperature.

Saturated aqueous solutions of *n*-butyl alcohol, ammonium nitrate, and ammonium chloride did not seem to peptize gliadin. Incidentally, the Hofmeister ion series is followed for SCN peptizes gliadin with ease; I, slowly; and NO₃ and Cl, not at all.

Acetamide-water peptizes gliadin, but upon dilution gliadin is not precipitated. It would seem that an acid-protein compound had formed in this case.

The well known investigations of Franklin and his students on ammonia as a solvent suggested that gliadin might be soluble in it. About 1 gm. of gliadin was introduced into a Faraday reaction tube and dehydrated at 60°C. and 2 mm. pressure. About 10 cc. of liquid ammonia, dried over sodium, were then introduced. The gliadin appeared not to dissolve, but immediately became yellow and gelatinous. It was allowed to stand in the closed tube at room temperature for 24 hours in contact with the ammonia. The supernatant, clear, colorless liquid was then decanted into the other leg, after which the cock was opened and the ammonia allowed to evaporate spontaneously. A translucent film which adhered to the glass was left in the leg which had contained the decanted liquid. However, this was not unaltered gliadin, for it would not dissolve in warm 50 per cent alcohol. The gliadin residue in the other leg had formed a horny vitreous mass which was somewhat yellowish and insoluble in warm 50 per cent alcohol. Evidently considerable alteration had taken place.

The observations on the peptization of gliadin presented in this paper have many interesting implications. It will be interesting to determine the critical peptization temperature of other prolamins. This may prove a new physical method for differentiating prolamins which cannot be distinguished readily by chemical methods.

There can be little question that Galeotti and Giampalmo (1908) determined, not the solubility of zein in alcohol-water

mixtures as they supposed but rather, the relative rates of solution (or peptization) of zein in these mixtures. This question should be reinvestigated.

Some of these new gliadin solvents may serve to separate gliadin from glutenin without irreversible alteration of the latter such as probably takes place in the present method of separating gliadin from glutenin with strong alcohol. In a few qualitative experiments gluten, prepared in the usual way, was extracted for 24 hours at 35–40°C. with solvents consisting of water containing 10 per cent isopropyl alcohol, 10 per cent *n*-propyl alcohol, 20 per cent *n*-propyl alcohol, 5 per cent isobutyl alcohol, and 20 and 50 per cent acetone. All of these solvents extracted considerable gliadin at this temperature. Further investigation might reveal that some of these solvents have no irreversible effect on the physical properties of glutenin and hence might be used for the isolation of pure glutenin. The fact that the gluten did not disintegrate in any of these solvents suggests that none of them peptizes glutenin.

Specific Rotation of Wheat Gliadin.

Many investigators have studied the specific rotation of gliadin in ethyl alcohol-water solutions. However, the observation of Mathewson (1906, *b*) that the specific rotation increases with the proportion of water in the solvent introduced a factor not considered by others. Kjeldahl (1892) employed as a solvent 55 per cent alcohol; Osborne and Harris (1903), 80 per cent alcohol; and others, 70 per cent alcohol. All observations found in the literature for $[\alpha]_D$ of isolated wheat gliadin in ethyl alcohol-water solutions are presented in Table VI.

The concentration of gliadin in the solutions employed by these investigators was determined in different ways. The results of Osborne and Harris (1903) were based on pure preparations. The concentration of gliadin was calculated from the nitrogen content of the polarized solutions, assuming $N \times 5.66 = \text{gliadin}$. Blish and Pinckney (1924) followed this method also. The concentration of the solutions employed by Mathewson (1906, *a*) in his earlier study was determined in the same way. He weighed out portions of his later preparation (1906, *b*) but since its nitrogen content was

17.47 per cent he corrected these results by the factor $\frac{17.66}{17.47}$.

Gróh and Friedl (1914) used weights of pure preparations with nitrogen contents of 17.49 and 17.57 per cent. Woodman (1922) did not report the nitrogen content of his preparation. "The amount of ash-free protein . . . was determined . . . by Kjeldahl, the nitrogen content of the gliadin samples being multiplied by the factor $\frac{100}{17.66}$."

TABLE VI.

$[\alpha]_D$ for Gliadin in EtOH-H₂O Solutions (Past Observations).

Observer.	EtOH by vol- ume in the mixed solvent.	$[\alpha]_D^*$	$[\alpha]_D^{20}$	$[\alpha]_D^{30}$	$[\alpha]_D^{40}$
	<i>per cent</i>				
Kjeldahl (1892).....	55	-92°			
Osborne and Harris (1903)...	80		-92.55° -91.9°		
Mathewson (1906).....	75		-88.2°		
" (1906).....	70		-93.1° -92.1° -91.8°	-92.9°	-91.3°
" (1906).....	60				-96.9° -96.4°
" (1906),.....	50				-98.6° -98.3°
Gróh and Friedl (1914).....	70		-91.0° -91.3°		
Woodman (1922).....	70	-93.6° -93.78°			
Blish and Pinckney (1924)...	70		-100.0°		
" " " (1924)...	70		-97.4°		
" " " (1924)...	70		-93.6°		

* Temperature not stated.

In addition to the values for $[\alpha]_D$ for solutions of gliadin isolated from flour, it is of interest to mention the values found by some investigators who did not attempt to isolate gliadin. Snyder (1904) found the value, -90°, based on the extract obtained directly from flour with 70 per cent alcohol. He calculated the

gliadin concentration from the total nitrogen content of the extract. Under various conditions of extraction and partial purification Lindet and Ammann (1907) found values ranging from -81.6° to -90.0° . Their results have never been duplicated by any other investigator.

All the observations were made with a Schmidt and Haensch half-shadow polarimeter. Readings other than those at 20°C . were made in jacketed tubes. The 20°C . readings were adjusted to $\pm 1.0^{\circ}$. It was found that a range in temperature of 3 degrees did not alter the reading perceptibly. White light filtered through a dichromate cell was used.

An investigation was first made of the effect on specific rotation of varying the proportion of alcohol in the solvent. Each of the five preparations of gliadin, of known moisture content, was employed. Enough gliadin was weighed into glass-stoppered, calibrated 25 cc. flasks to give a concentration of 3 to 4 per cent. The values obtained for specific rotation were calculated to the anhydrous basis. The proportions of alcohol in the solvent were 50, 60, and 70 per cent. By determination of the specific gravity of these solutions with a pycnometer, the concentration of alcohol was adjusted to within 0.1 per cent of the values indicated. The solvents and solutions were neutral to litmus paper. They gave a green color with brom-cresol purple and were neutral to phenolphthalein and methyl red. While the behavior of indicators in dilute alcohol differs from their behavior in aqueous solution, it seems certain that these solutions had a uniform hydrogen ion concentration which was not far from neutrality.

Specific rotation was determined on these solutions immediately and again after 12 to 48 hours. The final determinations were shown in Table VII. These average about 1 degree lower than the initial readings. The fact that they are 1 or 2 degrees lower than most of the values reported in the literature tends to confirm the suggestion made above that the accepted value for the nitrogen content of gliadin is too high. Most former determinations have been calculated, not to the basis of anhydrous preparation, but to a nitrogen content of 17.66 per cent.

An explanation was sought for the apparent slight change in rotation which took place during the interval between the initial and the final readings. It was thought that mutarotation or hys-

teresis might be involved, especially since gelatin in aqueous solution has been found by Trunkel (1910) to exhibit hysteresis. Solutions of gliadin were prepared at 20°C. by stirring the powder into 50, 60, and 70 per cent alcohol in small beakers. The exact concentration was disregarded. By vigorous stirring, solution was completed in 20 to 30 minutes and polarizations were made immediately. Observations were repeated later in the day and during the following day, but no distinctive change was noted.

The effect of temperature on rotation was next studied. Solu-

TABLE VII.

$[\alpha]_D^{20}$ for Gliadin in EtOH-H₂O Solutions (Observations Made on Solutions Which Had Been Standing for 12 to 48 Hours).

Gliadin preparation No.	EtOH by volume in the mixed solvent.		
	70 per cent.	60 per cent.	50 per cent.
	$[\alpha]_D^{20}$	$[\alpha]_D^{20}$	$[\alpha]_D^{20}$
1	-90.1°		
2 A	-90.5° -89.5°	-91.4° -90.8°	-90.3°
2 B	-89.7°	-91.6°	-89.0°
3 A	-89.4°	-90.1°	-91.1°
3 B	-90.0° -89.4°	-90.9° -91.2°	-89.1° -88.6° -89.9°
Average.....	-89.9°	-91.0°	-90.3°

tions of gliadin which had been prepared for some days were polarized at 20°, 30°, 40°, and 50°C. in jacketed tubes. The temperature was held to each level for approximately 30 minutes. The temperature was then lowered to 30° and to 20°C. under the same conditions. The concentration of these solutions at temperatures from 20° to 50°C. was found by determination of their increase in volume over this range in temperature, using a pycnometer. Observations on the specific rotation of these solutions are shown in Table VIII.

The evidence for hysteresis, shown in Table VIII, is unconvinc-

ing. While the behavior of the solution in 70 per cent alcohol suggested this phenomenon, the solution in 50 per cent alcohol, and that in 60 per cent alcohol, promptly returned to their earlier values. Accordingly, a final investigation was made. Solutions of gliadin were heated for 1 hour at 40°C. and then cooled rapidly to 20°C. and introduced into saccharimeter tubes. The first readings were made within 15 minutes and later readings were made after $\frac{1}{2}$, $1\frac{1}{2}$, and 4 hours. These readings of α were, for the solution in 50 per cent alcohol, -5.87° , -5.90° , -5.88° , and -5.88° ; for the solution in 70 per cent alcohol, -6.77° , -6.78° , -6.80° , and -6.78° . It seems justifiable to conclude that any change that does occur in $[\alpha]_D$ is not due to hysteresis.

TABLE VIII.
 $[\alpha]_D$ for Gliadin Preparation 3 A in EtOH-H₂O Solution at Various Temperatures.

	EtOH by volume in the mixed solvent.		
	70 per cent.	60 per cent.	50 per cent.
$[\alpha]_D^{20}$ (initial).....	-90.5°	-90.9°	-90.3°
$[\alpha]_D^{30}$ (upon first reaching 30°).....	-92.1°	-93.2°	-91.4°
$[\alpha]_D^{40}$ (" " " 40°).....	-93.7°	-95.8°	-94.2°
$[\alpha]_D^{40}$ (after 30 min. at 40°).....		-95.8°	-94.2°
$[\alpha]_D^{50}$			-96.9°
$[\alpha]_D^{30}$ (cooled from 40°).....	-92.7°	-93.1°	-91.5°
$[\alpha]_D^{20}$ (" " " 30°).....	-91.5°	-91.1°	-90.4°
$[\alpha]_D^{20}$ (after 18 hrs. at 20°).....	-90.3°		

The effect of acid and alkali upon the specific rotation of alcoholic gliadin solutions was investigated. A 20 cc. portion of a solution of gliadin in 60 per cent alcohol was pipetted into each of three small flasks. To one was added 5 cc. of distilled water; to the second, 5 cc. of N/14 hydrochloric acid; and to the third, 5 cc. of N/14 sodium hydroxide. The concentration of gliadin in these solutions was determined by polarization of the first, assuming $[\alpha]_D^{20}$ for gliadin in 50 per cent alcohol to be -90.0° . $[\alpha]_D^{20}$ was then determined for the other two solutions and found to be, for the acid solution, -100.5° ; and for the alkaline solution, -94.0° . These values were reached immediately and had not changed after 24 hours. The changes noted are in line with the observation of Bechhold (1919) that ionized albumin possesses greater power to

rotate light than neutral albumin. Equal volumes of the two solutions were than pipetted into a flask, and this solution was polarized. $[\alpha]_D^{20}$ was -94.0° .

Since it is possible that this higher value was due to the presence of sodium chloride resulting from the reaction of the gliadin chloride and of the sodium gliadinate, a study was made of the effect of a neutral salt on the specific rotation of gliadin. A gliadin solution was pipetted in 20 cc. portions into small flasks. Into the first were pipetted 5 cc. of 50 per cent alcohol and the concentration of gliadin was determined accurately by polarization. Into the second were pipetted 5 cc. of a solution of lithium chloride in 50 per cent alcohol of such concentration as to give a lithium chloride-gliadin ratio of 2:1000. Two other solutions were prepared in the same way, one with a lithium chloride-gliadin ratio of 10:1000 and the other, 25:1000. The values for α for these three solutions at room temperature were -4.45° , -4.46° , and -4.45° . Apparently lithium chloride does not affect the specific rotation of gliadin in the above described proportions. No further study was made of the reason for the high rotation noted above. It is due possibly to the different strengths of the acid and basic dissociation constants of gliadin, or possibly simply to the fact that when equivalent amounts of gliadin chloride and sodium gliadinate are mixed, the neutralization is incomplete.

Some observations have been made on the specific rotation in other solvents. The value for $[\alpha]_D^{20}$ in 30 per cent *n*-propyl alcohol is -97.7° ; in 40 per cent, -98.4° ; in 50 per cent, -98.6° ; and in 60 per cent, -98.1° . There is, therefore, no conclusive evidence of any change in rotation within this range of *n*-propyl alcohol-water mixtures.

It has been noted above that Spiro suggested that the dissolving of proteins in aqueous urea solutions may be explained by the formation of alkali proteinate, the urea acting as an alkali. Some observations on the specific rotation of gliadin in solvents containing urea render this conclusion doubtful. The specific rotation of gliadin in 30 per cent aqueous urea solution is -116.5° . After heating in a sealed tube at 37°C . for 7 days this value was unchanged. One would expect if an alkali albuminate had been formed that some racemization would have taken place.

SUMMARY.

Gliadin preparations of unusual purity have been obtained. The essentially new features of the method of preparation followed are: (a) precipitation in aqueous solution is made with vigorous shaking; and (b) lithium chloride is used instead of sodium chloride to promote precipitation. The first modification has the advantage that the gliadin is precipitated in a foam and hence can be washed thoroughly. The use of lithium chloride makes a practically ash-free preparation possible on account of the solubility of this salt in ether and alcohol. The five gliadin preparations obtained had an average nitrogen content of 17.54 per cent.

It has been found that gliadin may be irreversibly altered under certain conditions with suitable alcohol-water mixtures. The significance of this observation in connection with the determination of gliadin and the isolation of glutenin is discussed.

When a solution of gliadin in alcohol-water is cooled, there is a definite temperature at which turbidity appears. This turbidity temperature varies with the proportion of alcohol in the solvent, but is practically independent of the concentration of gliadin. It may therefore be called a critical peptization temperature.

Gliadin is peptized at room temperature in a somewhat narrow range of methyl alcohol-water mixture; in a somewhat wider range of ethyl alcohol-water mixtures; and in a much wider range of *n*-propyl alcohol-water mixtures. The critical peptization temperatures of gliadin in each of these mixtures were determined.

There appears to be no upper limit to the solubility of gliadin in these mixed solvents above the critical temperature. Thus gliadin readily formed concentrated solutions in 10 per cent ethyl alcohol at 50°C. and a solution of gliadin in 70 per cent *n*-propyl alcohol was evaporated to a clear, gelatin-like film at 50°C. without development of turbidity or evidence of precipitation.

The critical peptization temperature curves of the five gliadin preparations in ethyl alcohol-water mixtures are similar in form. The lowest temperature on each curve corresponds to a concentration of about 60 per cent alcohol by volume in the solvent.

Many new solvents for gliadin have been found.

The specific rotation of pure gliadin in ethyl alcohol-water solutions has been redetermined. With solvents containing 70, 60,

and 50 per cent alcohol by volume, $[\alpha]_D^{20}$ was -89.8° , -91.0° , and -90.3° , respectively. These figures indicate a maximum rotation in 60 per cent alcohol instead of, as Mathewson reported, a rotation which increases with the percentage of water in the solvent.

These values were determined on solutions which had been prepared at 40°C . and which had been standing for 12 hours or longer. Readings made on these solutions immediately after preparation gave rotations which averaged about 1 degree higher. This apparent change in rotation seemed to be an elusive one, for it was impossible to determine the exact conditions under which it took place. Solutions of gliadin prepared rapidly by vigorous stirring at room temperature were polarized immediately and at subsequent times, but showed no mutarotation. Nor was there definite evidence of hysteresis as stable gliadin solutions were cooled from 50°C . to 20°C .

In solvents containing 70, 60, and 50 per cent alcohol by volume, the specific rotation of gliadin increased considerably over a temperature range from 20°C . up to 50°C .

The values found for the specific rotation of gliadin are 1 or 2 degrees lower than most values in the literature. This difference is partly due to the fact that weighed portions of pure preparations were used. Most other investigators have calculated their results to 17.66 per cent of nitrogen, whereas evidence is here presented that this value is too high. The fact that freshly prepared solutions appear to possess slightly higher rotatory power may also have contributed to the higher figures of other investigators.

The specific rotation of gliadin in *n*-propyl alcohol-water at 20°C . averaged -98.2° and was not found to vary significantly with the proportion of water in the solvent.

The specific rotation of gliadin in a 30 per cent aqueous urea solution was -116.5° . Heating this solution at 37°C . for 7 days did not alter the specific rotation. The formation by urea and gliadin of an alkali albuminate seems unlikely since no racemization took place.

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STUDIES IN CARBOHYDRATE METABOLISM.

II. INVESTIGATIONS INTO THE MUTAROTATION OF β -GLUCOSE UNDER VARIOUS CONDITIONS.

BY CHRISTEN LUNDSGAARD AND SVEND AAGE HOLEØLL.

(From Medical Clinic A, University of Copenhagen, Copenhagen, Denmark.)

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INTRODUCTION.

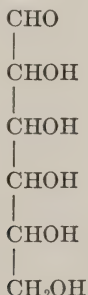
In a previous contribution,¹ we have shown that a glucose solution with the specific rotatory value of $+52.5^\circ$ always shows a lower rotatory power after the addition of insulin and fresh muscle tissue than that which corresponds to the reduction value. According to our results this fact could either indicate a shifting of the equilibrium between α - and β -glucose in the mixture, with the formation of β -glucose at the expense of α -glucose, or a production of an entirely new form of glucose with a low specific rotatory power.

As this question is of great importance both theoretically as well as practically, we have continued our investigations into the problem.

Remarks on the Chemistry of Glucose.

Before passing on to report our results, we will, in order to make them intelligible, give a short review of the most recent chemical views regarding the constitutional formula of glucose and the possibility of the occurrence of several stereoisomeric forms.

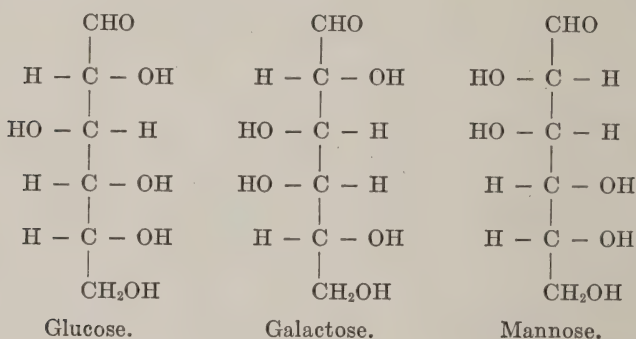
On inspecting the ordinary formula for an aldohexose:



¹ Lundsgaard and Holtøll (1924-25), referred to as Paper I of this series.

it will be seen that the 4 middle carbon atoms are asymmetric, an asymmetric carbon atom being one whose four valencies are united with 4 *different* atoms. The presence of asymmetric carbon atoms in a formula shows that there must be several stereoisomeric compounds of this constitution. If there are n asymmetric carbon atoms the number of stereoisomeric compounds is 2^n .

This shows that there must be $2^4 = 16$ stereoisomeric compounds which are aldohexoses. Of these, 14 have hitherto been prepared. The best known are glucose, galactose, and mannose, and their formulas can be written as follows:



The presence of the asymmetric carbon atoms is responsible for the power of the given sugar to rotate polarized light. With respect to this characteristic practically all reducing kinds of sugar show the phenomenon which is termed *mutarotation* which does not seem to be explicable on the basis of the other formulas.

Dubrunfaut in 1846 was the first to find that a fresh glucose solution had nearly twice as great a rotatory power as it had the next day, a phenomenon which he called mutarotation. The mutarotation of glucose was afterwards thoroughly investigated by a number of different workers (Levy, Trey, Tanret, etc.).

It appears that mutarotation is hastened by heating and is momentarily inhibited by the addition of small amounts of alkali. The addition of acids has also an accelerating influence on the process.

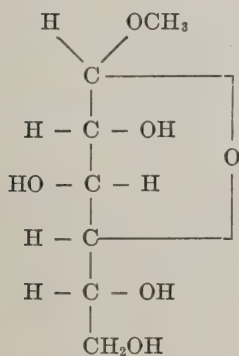
In the course of time various explanations of the phenomenon of mutarotation have been put forward. Thus it has been supposed that the freshly prepared solution contains molecular aggregates of special constitution which gradually break down into simple molecules with a lower rotatory power. It has been shown by cryosecopy, however, that the molecular weight is not altered with mutarotation and only *intramolecular* changes can, therefore, take place. Nor can the addition and subsequent splitting off of water explain the mutarotation, as the latter also occurs in absolute alcohol. One has, therefore, to assume the presence of

several isomeric compounds which can change into one another. This view was made highly probable by Tanret's preparation of a form of glucose which immediately after solution showed a low rotatory value and only gradually attained the equilibrium point. Thus there are two forms of glucose which have all the following properties in common: appearance, solubility, melting point, fermentative power, reduction power, and other chemical reactions, and only differ in their power of rotating polarized light.

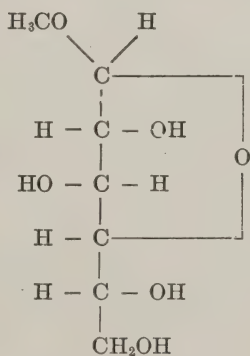
The occurrence of two such forms of glucose cannot be reconciled with the above constitutional formula. An explanation of mutarotation has, however, been arrived at by studying the reactions of methylglucoside. E. F. Armstrong showed that when the two methylglucosides prepared by Emil Fischer were decomposed with the help of enzymes a different result was obtained according to whether one started with the strongly dextro-rotatory glucoside or with its isomer, the levorotatory glucoside. In the first case a sugar solution was obtained with a rotatory power greater than $+52.2^\circ$. In the second case a solution with a lower rotatory power was obtained. He concluded, therefore, that the mutarotation was due to the presence of two forms of sugar whose isomers must be explained in the same way as the two glucosides.

Jungius has since investigated the conditions under which the methylglucosides change into one another in the presence of hydrochloric acid, and has shown that this process is analogous with the phenomenon of mutarotation except that it proceeds more slowly. The isomers of the glucosides and the two forms of glucose must therefore be explained in the same way.

It has been shown by different methods and by different investigators (E. Fischer, Irvine) that the following formulas are valid for the methylglucosides.

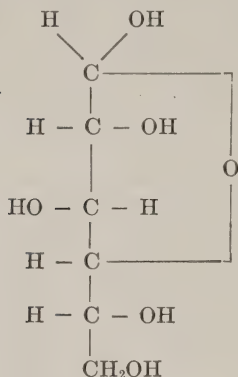


α -Methylglucoside.
 $+158.2^\circ$



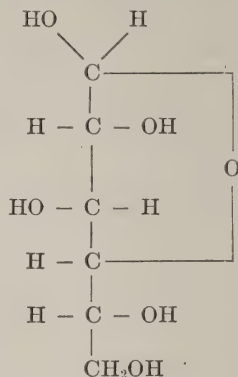
β -Methylglucoside.
 -31.9°

The formulas for α - and β -glucose are therefore as follows:



α -Glucose.

About $+110^\circ$.



β -Glucose.

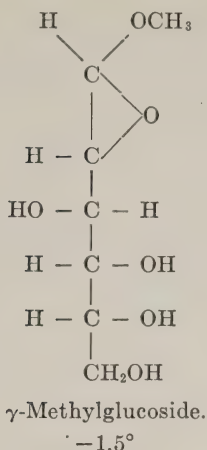
About $+19^\circ$.

In these formulas the hydrogen in the fourth carbon atom's OH group is combined with the carbonyl group's O atom. The carbon in the carbonyl group thus has a free valency which is then united to the O atom in the original hydroxyl group with the formation of a ring. *In this way there is still formed an asymmetric centre, so that the occurrence of two forms of glucose with different rotatory powers becomes possible.*

The mutarotation must, therefore, be due to the H and OH groups changing position from one side of the ring to the other until equilibrium between the amount of α - and β -glucose, corresponding to the specific rotatory value $+52.2^\circ$, is attained. The accuracy of the formulas for α - and β -glucose is further confirmed by more recent investigations, for instance those of Pictet and of Bøeseken who have each by different methods strengthened the validity of the formulas.

It may, moreover, be remarked that the above formulas also explain the fact that the glucose in certain respects is less active than other substances which contain the free aldehyde group CHO.

About the same time Emil Fischer and Irvine and coworkers found that a third methyl derivative can be prepared by methylation of glucose, with quite other characteristics; namely, entirely different rotatory power and much quicker and stronger reduction power. E. Fischer gave it the name γ -glucoside and showed that here the ring was formed between the first 2 carbon atoms.



It is clear that the presence of the ring and, therefore, the production of a new asymmetric carbon atom creates the possibility for the existence of two stereoisomeric forms, so that the γ -methylglucoside must probably be regarded as a mixture of two such forms.

The proof is thus furnished that the ring, besides being formed between the first and fourth carbon atoms, can also occur between the first and second, and there is naturally a theoretical possibility for combinations where the ring is formed between the first and third or the first and fifth carbon atoms. All these combinations again must be assumed to occur in the stereoisomeric forms.

As it is proved that there are more than two methyl combinations of glucose the probability is that glucose also occurs in other forms than the α and β forms.

It must be emphasized, however, that such forms have never been prepared, but various investigators have assumed their existence from certain experimental observations. Thus, Hewitt and Pryde found that a glucose solution in equilibrium underwent changes in the intestinal canal of the living animal, in that a rapid downward mutarotation of the sugar solution to values much lower than $+52.5^\circ$ occurred. Different values were measured below the specific rotatory value $+19^\circ$ of β -glucose. After removal from the intestine a slow upward change in the mutarotation to a permanent value, which corresponded to α - and β -glucose in a state of equilibrium, took place. Further, they found that the glucose in the solution immediately after removal from the intestinal canal reacted more strongly in certain chemical respects than ordinary glucose. Hewitt and Pryde assume the existence of a glucose form with low rotatory power from their experiments which perhaps is even levorotatory. They call it γ -glucose and assume that the ring, as in the formula of γ -methylglucoside, is formed between the first and second carbon atoms.

Later, Winter and Smith carried out comparative investigations into the reduction and rotation powers of the blood sugar in normal persons and diabetics, from which they concluded that the blood sugar in normal persons occurs in the γ form.²

EXPERIMENTAL.

The reason that we did not directly explain the results of our investigations, namely that the addition of insulin and fresh muscle tissue to a sugar solution produced a decrease in the specific rotatory power, by assuming the formation of a new form of glucose with a low specific rotatory power, is because, in contrast to Hewitt and Pryde, and Winter and Smith we had never measured any specific rotatory power lower than the $+19^\circ$ of β -glucose. The diminished rotation observed could also be accounted for by a displacement of the equilibrium between α - and β -glucose in the mixture. In order to find out which of these possibilities is the correct one, *we have compared the course of the mutarotation for the glucose transformed by insulin and muscle with the course of the mutarotation for chemically prepared β -glucose.*³

From the results in our first communication it appeared that the mutarotation proceeded very slowly for the form of glucose produced under the influence of insulin and muscle. Not until it had stood for 48 hours at room temperature was the specific rotatory power usually constant and equal to that of α , β -glucose. This slowness in the course of the mutarotation which we have found in our experiments agrees with the investigations of Winter and Smith, but not, however, with those of Hewitt and Pryde, in whose experiments the mutarotation proceeded much more quickly and was finished in about 1 hour. We shall discuss this difference in more detail later.

² In the first paper of this series we wrote that Winter and Smith "had found that insulin affected pure solutions of α , β -glucose." We regret that this quotation was a misunderstanding on our part since Winter and Smith had stated that insulin did not act upon glucose. Our results in this respect were, therefore, in full confirmation of their findings.

³ The chemically prepared β -glucose which, like the insulin preparation employed, was furnished by "Det danske Medicinal-Kompagni" and was kindly prepared for us by the Laboratory Director, Mr. Gad-Andresen.

Course of the Mutarotation in a Solution of β -Glucose.

As far as ordinary glucose is concerned the mutarotation phenomenon in the literature has been exclusively studied with α -glucose as the starting point. It was, however, to be expected that the transformation of β -glucose to the equilibrium point proceeded in the same way as that of α -glucose.

This view was confirmed in the following manner. In a glucose solution with the specific rotatory power of $+52.5^\circ$ there must be, in accordance with what has previously been said, 36 per cent of

TABLE I.

Mutarotation for 10 Per Cent β -Glucose in Distilled Water at 18°C .

Time.	Specific rotatory power.
<i>min.</i>	
3	24.5°
10	27.5°
18	30.1°
25	33.9°
36	37.3°
48	40.0°
63	42.6°
75	44.2°
93	46.2°
105	47.6°
129	49.7°
168	52.2°
180	52.5°
195	52.5°

α -glucose with the specific rotatory power of $+110^\circ$ and 64 per cent of β -glucose with the specific rotatory power of $+19^\circ$. A mixture was, therefore, prepared which contained α - and β -glucose in these proportions. On dissolving this mixture in water the specific rotatory power $+52.5^\circ$ was immediately observed and it kept constant at this value.

The course of the mutarotation in a solution of β -glucose was next examined. In Fig. 1 the course of the mutarotation in a 10 per cent solution of β -glucose in distilled water at a temperature of 18°C . will be seen. Besides dissolving a definite quantity of β -glucose in a measured volume of distilled water, the concentra-

tion of the solution was always determined by analysis of the reduction power after the experiment. The rotation values were read at suitable intervals during the experiment until the mutarotation was proved to have come to an end by several successive readings showing the same value.

The specific rotatory power was calculated from the rotation values read, and this value is entered both on the curve and in

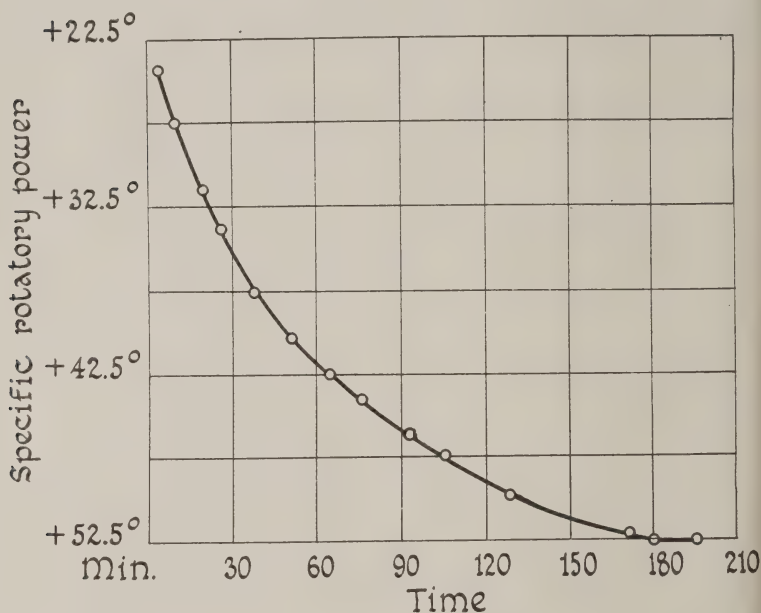


FIG. 1. The course of the mutarotation in a neutral solution of 10 per cent β -glucose at 18°C.

Table I. The first determination was made as rapidly as possible; it was usually taken after 3 minutes had passed. The rotation values are the means of ten readings and the time recorded is the middle point of the interval which was required for the reading. It will be seen from Fig. 1 and Table I that the mutarotation on dissolving 10 per cent β -glucose in distilled water at 18°C. was finished after 3 hours.

*Influence of Glucose Concentration on the Course of the
Mutarotation.*

The course of the mutarotation for a series of different concentrations of β -glucose was next examined, varying from 0.5 to 40 per cent, under the same conditions; that is to say, a solution of

TABLE II.

*Course of the Mutarotation for the Concentrations 0.5 to 40 Per Cent β -Glucose
in Distilled Water at 18°C.*

Time.	0.5 per cent.	1.0 per cent.	2.0 per cent.	4.0 per cent.	10.0 per cent.	20 per cent.	40 per cent.
<i>min.</i>							
5	25.2°	25.3°	25.5°	26.0°	25.9°	25.8°	25.4°
10	26.9°	27.5°	27.3°	28.0°	27.7°	27.2°	27.5°
15	29.1°	30.2°	30.3°	30.0°	30.0°	29.4°	30.6°
30	35.5°	35.9°	36.0°	35.4°	35.3°	36.1°	35.8°
60	42.3°	42.3°	42.1°	42.2°	42.2°	42.3°	42.2°
90	45.7°	46.0°	46.0°	46.1°	46.0°	45.9°	45.9°
120	48.8°	48.8°	49.0°	48.9°	49.1°	49.0°	49.1°
150	51.3°	51.2°	51.2°	51.3°	51.2°	51.4°	51.3°
180	52.5°	52.5°	52.5°	52.5°	52.5°	52.5°	52.5°

TABLE III.

Influence of Temperature on the Course of the Mutarotation.

Time.	18°C.	37°C.
<i>min.</i>		
5	25.9°	29.2°
10	27.7°	33.1°
15	30.0°	37.3°
30	35.3°	44.0°
60	42.2°	50.4°
90	46.0°	52.5°
120	49.1°	
150	51.2°	
180	52.5°	

β -glucose in distilled water at 18°C. All the curves showed a course absolutely identical with Fig. 1. This will be most easily seen from the values for the specific rotation at the different times, which are recorded for all the concentrations in Table II. In the calculation of these experiments the slight displacement of the

specific rotatory power caused by variations in the concentration of glucose is neglected.

Influence of Temperature on the Course of the Mutarotation.

The influence of the temperature on the mutarotation is shown in Fig. 2 where the mutarotation at the temperatures 18° and 37°C. is given. The accelerating influence of the higher temperature

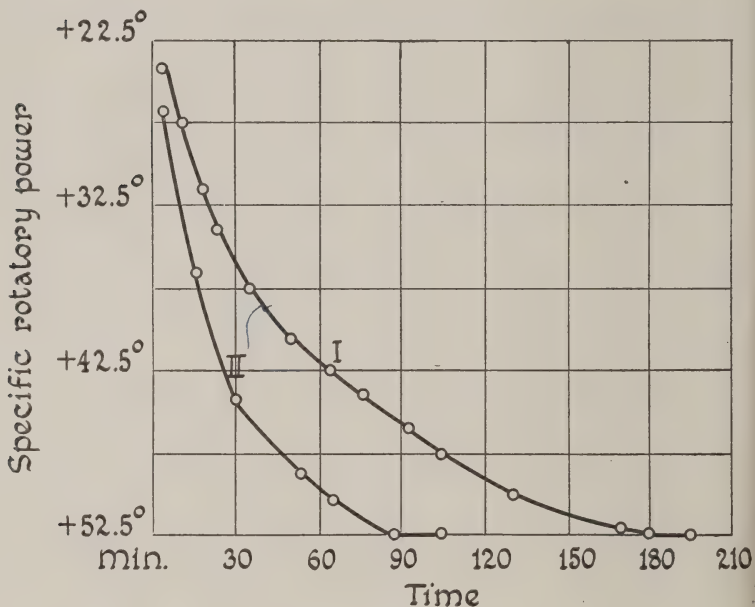


FIG. 2. The influence of temperature on the course of the mutarotation. Curve I, 18°C.; Curve II, 37°C.

is clearly seen from the curves and the figures for the specific rotatory value in Table III.

In the last experiment the β -glucose was dissolved in warm water at 37° and the polarization tube was kept in a thermostat at this temperature between the readings. Control experiments showed that the temperature in the liquid during the reading did not sink below 36.5°.

Influence of Hydrogen Ion Concentration on the Course of the Mutarotation.

A very important point in the mutarotation process is the reaction of the solvent. The mutarotation was, therefore, determined for a number of solutions of different reactions, the pH for the different liquids varying between 1.0 and 12.0.

In Fig. 3 the mutarotation is given for the solutions whose pH was between 1.0 and 7.0. It will be seen from the curves and the corresponding values of the specific rotatory power in Table IV that no difference was found in the course of the mutarotation for the liquids having a pH between the neutral point and 4.0. With pH 1.0 and 2.0 a marked acceleration of the process was found, however.

TABLE IV.

Influence of Reaction on the Course of the Mutarotation.

Temperature 18°. pH from 1.0 to 12.0.

Time. min.	pH								
	1.0	2.0	4.0	5.0	6.0	7.0	9.0	10.0	12.0
3	26.4°	25.4°	24.6°	24.5°	24.4°	24.5°	52.5°	52.5°	52.5°
15	41.2°	32.9°	30.0°	29.6°	30.3°	29.8°			
30	48.1°	38.7°	35.8°	36.0°	36.1°	35.7°			
60	51.3°	46.2°	42.1°	42.3°	42.3°	42.2°			
90	52.5°	50.3°	46.0°	45.8°	46.0°	46.0°			
120		52.5°	49.0°	49.1°	49.2°	48.7°			
150			51.2°	51.4°	51.0°	51.1°			
180			52.5°	52.5°	52.5°	52.5°			

If we now look at the results with solutions in an alkaline liquid quite different values are found as will also be seen from Table IV. Already with a solution in a liquid with a pH 9.0, the mutarotation had come to an end before the first reading, after the process had been proceeding for 3 minutes, was taken.

We have now investigated the course of the mutarotation for a chemically prepared β -glucose under the same conditions as those which apply to the transformed glucose in our experiments, and we have thus obtained the means for comparing the course of the mutarotation for the two forms of glucose from which we can conclude whether they are two different substances or not.

As will be remembered from our first contribution the action of insulin and muscle tissue on the glucose solution took place at 37° , while the dialysis took place at room temperature. The dialysate was afterwards kept at this temperature until the mutarotation was finished. In a number of experiments we also determined the reaction of the dialysate. It was found that the pH only varied between 6.70 and 7.00.

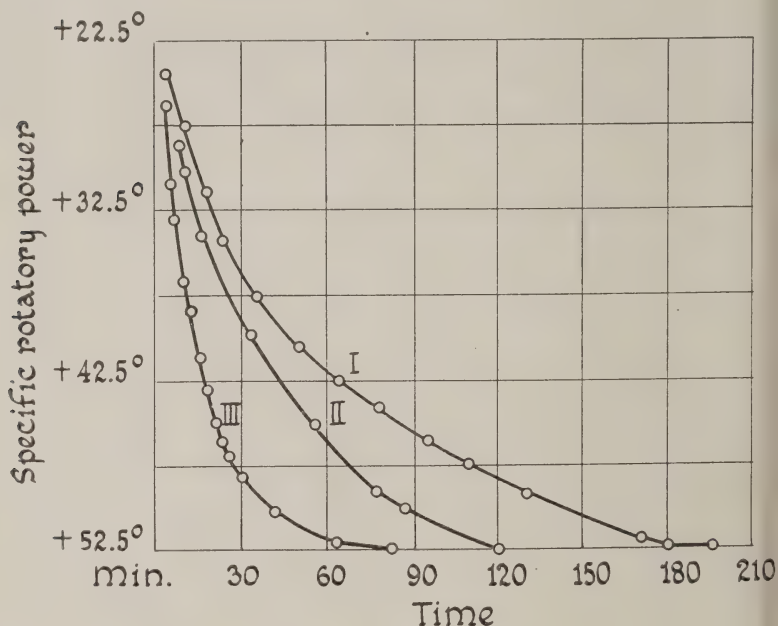


FIG. 3. The influence of reaction on the mutarotation. Curve I, pH 4.0, 5.0, 6.0, and 7.0; Curve II, pH 2.0; Curve III, pH 1.0. 10 per cent β -glucose. Temperature 18°C .

On comparing the results for the course of the mutarotation under the same conditions for β -glucose with the form of glucose prepared by the biological method it is obvious that there is a considerable difference, in that the mutarotation in the former was finished in a few hours while that of the latter, under similar conditions, was only finished after 48 hours.

Experiments on the Stabilization of β -Glucose.

Even if the results obtained are absolutely against the substance produced being β -glucose there is still a fact which must be considered. We imagine that the muscle and insulin, besides displacing the equilibrium between α - and β -glucose, exert a *stabilizing influence* on any β -glucose thus formed. This point was investigated by adding muscle and insulin to a freshly prepared solution of β -glucose and determining its mutarotation.

A fresh 2 per cent solution of β -glucose was made, to which 15 gm. of fresh muscle and 20 units of insulin were added. After the action had gone on for 2 hours at 37°C . a sample was removed and put in the collodion membrane at room temperature, and the dialysate was examined after $1\frac{1}{2}$ hours. The sugar in the dialysate, which therefore was examined $3\frac{1}{2}$ hours after the β -glucose was dissolved, showed a specific rotatory value of $+47.25^{\circ}$ and it was found that this value only rose slowly until, after 48 hours, it had reached $+52.5^{\circ}$.

The diminution in the rotatory value observed in this experiment is precisely the same as was found in the same interval in a 2 per cent α , β -glucose solution in the presence of the same amount of muscle and insulin as was used in this experiment. The conclusion must therefore be that the addition of muscle and insulin *has had no effect on the mutarotation of the β -glucose*, but that the latter had come to an end before the dialysate was examined, as it should, according to Fig. 2, after 2 hours at 37° . Further, the experiment shows that the decrease in the specific rotatory angle which occurs on the addition of insulin and fresh muscle tissue, under otherwise similar conditions, is the same whether we began with α , β -glucose or with β -glucose.

From our experiments published in the earlier communication it is clear that the mutarotation also proceeds very slowly in a mixture of glucose, insulin, and muscle tissue even at the time when the muscle tissue has lost its power of transforming the glucose. This shows us that in these experiments on possible stabilization we are able to employ muscle tissue which no longer possesses the power of transforming glucose. Thus an action on β -glucose would be more simple and the results more easily interpreted.

As reported in our earlier work, it was found that muscle tissue on being kept for 2 hours in physiological salt solution lost its power to transform glucose and we were able to avail ourselves of this in the following experiments.

First of all the following experiment was made. To 200 cc. of a 2 per cent β -glucose solution, insulin and 15 gm. of muscle were added which had been kept in physiological salt solution for 2 hours. After 2 hours at 37°C. it was dialyzed, and the dialysate was examined after 1½ hours. The specific rotatory value was then found to be +52.5°; in other words, the mutarotation had

TABLE V.

Course of Mutarotation for Pure β -Glucose and for β -Glucose to Which Muscle and Insulin Have Been Added.

Time.	Pure β -glucose solution.	1 per cent β -glucose + insulin + muscle.	2 per cent β -glucose + insulin + muscle.
<i>min.</i>			
10	33.5°		
20	39.6°		
30	43.2°		
60	47.3°		
75	48.6°		
84	49.2°	49.2°	49.1°
95	50.1°	49.9°	50.0°
105	50.7°	50.7°	50.8°
117	51.4°	51.2°	51.5°
126	51.9°	52.0°	51.9°
133	52.2°	52.3°	52.2°
144	52.5°	52.5°	52.5°

ceased, as according to Fig. 2 it would have done if the muscle and insulin had not been added. No influence on the course of the mutarotation could, therefore, be detected.

In a final experiment the standing at 37° was shortened to 20 minutes and the dialysis to an hour. It was thus possible to obtain the sugar for investigation at a time when the mutarotation had not yet come to an end and we could then determine with certainty whether the addition of insulin and muscle had any influence on the rapidity of this process. First of all the mutarotation curve had to be determined for β -glucose kept for 20 minutes at 37°C.

and then at 18°. This is represented by Fig. 4 and the specific rotatory values are entered in the second column of Table V.

In the actual experiment 1 and 2 per cent β -glucose were employed to which were added insulin and 15 gm. of muscle which had been kept for 2 hours. The specific rotatory values are recorded in Table V and shown in Fig. 4. These values quite coin-

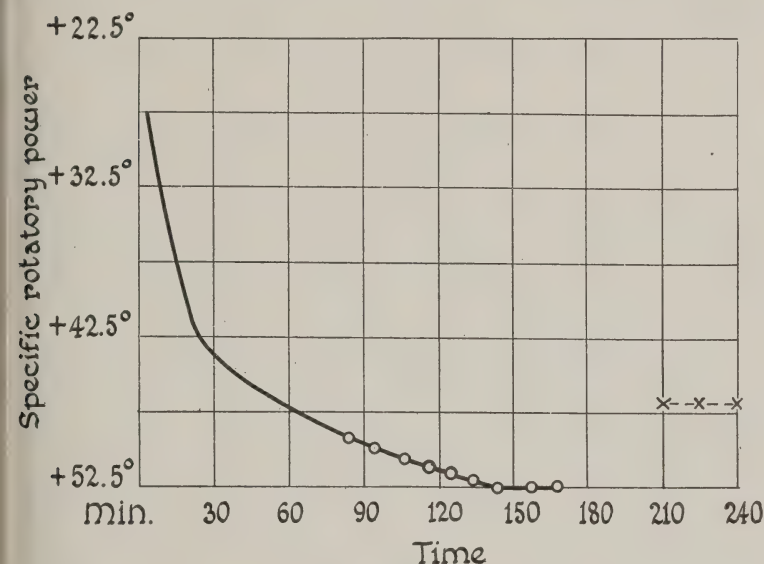


FIG. 4. The course of the mutarotation in a solution of β -glucose after the addition of insulin and muscle tissue.

— Curve of β -glucose kept for 20 minutes at 37°C. and then at 18°C.

o—o—o Values for β -glucose to which inactive muscle and insulin have been added, kept for 20 minutes at 37°C. and then at 18°C. This curve accurately coincides with the last part of the first.

x---x---x Values for β -glucose to which fresh muscle and insulin have been added, which have been allowed to act for 2 hours at 37°C. and then at 18°C.

ide with the values for the pure solution and it is therefore proved that the addition of muscle and insulin has no effect on the rapidity of the course of the mutarotation in the case of β -glucose.

The slow mutarotation which we found for the glucose transformed in the presence of insulin and muscle cannot, therefore,

be due to any stabilizing influence of the muscle or insulin on any possible β -glucose formed, and we can therefore state as the result of our investigations: *The decrease in the specific rotatory value which we have demonstrated in a solution of α , β -glucose to which fresh muscle tissue and insulin have been added is not produced by a displacement of the equilibrium between α , β -glucose in the mixture, but is due to the formation of a new form of glucose with a low specific rotatory power.*

DISCUSSION.

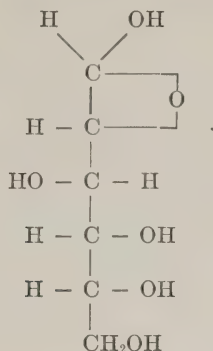
It was *a priori* probable that our experiments would lead to the result obtained. For if the first effect of the insulin on the transformation of glucose in the organism is supposed to be the conversion of the glucose into a new form of glucose which can then be combusted, we should have to assume the formation of an entirely new form of glucose with much more active properties than α - and β -glucose.

The production of an increased amount of β -glucose would not explain the action of the insulin. The constitution of α - and β -glucose must be taken to be exactly the same as regards their transformation in the organism. It must, moreover, be remembered that in the form of sugar which passes through the intestinal wall there is 64 per cent of β -glucose. If β -glucose was a form of glucose which diabetics also could combust they would always be able to make use of at least about two-thirds of the carbohydrate consumed. We know, however, that this is far from being so in the more severe cases of diabetes.

The reason that we carried out all these experiments in spite of the fact that on the face of it it was improbable that the diminution in rotatory power which we found could be ascribed to an increase in the amount of β -glucose, was that as we had previously shown there was a possibility of the presence of a number of stereoisomeric combinations of glucose, and for each of these forms which could be excluded with certainty from consideration, the possibilities there were to choose between would be diminished.

It only now remains to discuss the possible constitution of this substance. Hewitt and Pryde, and later, Winter and Smith

assumed, as has been mentioned, that it was a glucose with the following formula.



If this compound existed it would possess considerably greater chemical characteristics than α - and β -glucose on account of the bonds between the first and second carbon atoms.

We have previously stated that the mutarotation in the case of the compound found by Hewitt and Pryde proceeds much more quickly than in Winter and Smith's experiments. We do not, however, consider that this precludes its being the same substance as that we have to deal with in the latter experiments. While Winter and Smith had their substance in a neutral or slightly acid solution, Hewitt and Pryde, on account of the admixture of intestinal secretion, had their substance in an alkaline solution, which fact is undoubtedly sufficient to explain the difference in the course of the mutarotation.

We think, however, that there are other possibilities than the formation of the hypothetical γ -glucose. As is known, the old formula in which the glucose was represented as an aldehyde was abandoned in favor of the newer formulas given, partly because an explanation of the phenomenon of mutarotation was then possible and partly because certain important aldehyde reactions cannot be produced in glucose solutions. There seems now to be a possibility that the conversion from α -glucose to β -glucose always takes place with the aldehyde formula as an intermediate link and that besides the α and β combinations there is always a very small amount of glucose present as aldehyde in a glucose solution. The added insulin and muscle may be imagined to act by causing the transformation of a larger part of the α , β -glucose into aldehyde

glucose. The latter compound must have much more active chemical properties and could very well be expected to be burnt up in the organism. We have experiments in progress on this problem.

SUMMARY.

1. On comparing the course of the mutarotation of chemically prepared β -glucose with that of glucose transformed by insulin and muscle tissue, a well marked difference is found.

2. It is shown that this difference cannot be explained by any stabilizing influence of the muscle and insulin on β -glucose, whose reversion to α , β -glucose takes place with the same rapidity with and without this addition.

3. The diminution in rotatory power of a glucose solution found in our earlier experiments on adding insulin and fresh muscle tissue cannot therefore be due to an increase in the amount of β -glucose in the mixture, but must be caused by the presence of a new form of glucose with a low rotatory power.

4. The possible constitution of this as yet unknown form of glucose is discussed. Besides the possibility that it may be the so called γ -glucose, the suggestion is put forward that it may be due to the formation of glucose containing the aldehyde group in a free connection. We propose that the term *new-glucose* be employed temporarily for the form of glucose prepared by us in the manner described.

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STUDIES IN CARBOHYDRATE METABOLISM.

III. INVESTIGATIONS INTO THE NATURE OF THE GLUCOSE IN THE BLOOD OF NORMAL INDIVIDUALS.

BY CHRISTEN LUNDSGAARD AND SVEND AAGE HOLBØLL.

(From Medical Clinic A, University of Copenhagen, Copenhagen, Denmark.)

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INTRODUCTION.

In our previous papers,¹ we showed that by adding insulin and fresh muscle tissue *in vitro* to a solution of α,β -glucose a transformation of part of the glucose into a new, hitherto unknown, form of glucose takes place. This form, which for the present we have called *new-glucose*, is characterized by a lower specific rotatory power than that of α,β -glucose ($+52.5^\circ$). As we advanced the hypothesis that this form of glucose was the first step in the transformation of α,β -glucose in the organism, it is of importance to know whether the same form of glucose can be detected in the blood of persons with normal carbohydrate metabolism. Our knowledge of new-glucose was limited to the fact that its specific rotatory power was lower than that of α,β -glucose and that its transformation into α,β -glucose took place very slowly, the mutarotation lasting 48 hours at room temperature. The possible detection of new-glucose in the blood of normal persons would, therefore, have to be carried out by determining the specific rotatory power of the glucose in the blood, with simultaneous determinations of the reducing power and rotatory power of the blood sugar.

Earlier Investigations into the Specific Rotatory Power of the Blood Sugar.

Before Ivar Bang's introduction of the micro methods, particularly for the estimation of the blood sugar, polarization determinations were used

¹ Lundsgaard and Holbøll (1924, 1925).

for the quantitative estimation of glucose in blood. Such analyses were made by B. Oppler and by Michaelis and Rona, all of whom found fairly good agreement between the rotatory values and the reduction values of glucose in the blood of normal persons and animals. Since, however, their method of providing the glucose in suitable form for the polarization determination was such (evaporation of the protein-free filtrate) that any new-glucose present would be converted into α,β -glucose, their investigations have no significance in this connection.

In 1922, as mentioned in our first paper, the English workers. Winter and Smith, published some work from Hopkins' laboratory, in Cambridge, from the results of which they conclude that the glucose in the blood of normal animals and man occurs in a form which is different from ordinary α,β -glucose. The mode of procedure in their experiments was as follows: Immediately after drawing 100 cc. of blood the proteins were precipitated by Folin and Wu's method with phosphotungstic acid. The filtrate was desiccated in a vacuum at a temperature which did not exceed 40°C. Alcohol was then added to precipitate any possible trace of protein bodies still remaining and the filtrate from this was again desiccated in a vacuum. Finally the dried residue was dissolved in 10 to 20 cc. of water and the reduction and rotation values of this solution were determined. In the most favorable cases the technique described could be completed in 6 hours. They found in normal animals and man a lower value for the rotatory power than for the reduction power in all cases. If the specific rotatory power is calculated from their results, values between $+13^\circ$ and $+42^\circ$ are found. On continuing the determination of the rotatory power it was found that it slowly rose and in the course of 3 to 4 days coincided with the reduction power. Further, they found that the glucose in the filtrate at first had a stronger decolorizing action on a solution of potassium permanganate than some time later.

Winter and Smith conclude from their results that the glucose in the filtrate, at any rate, partly occurs as the so called γ -glucose, the hypothetical form of glucose first discussed by Hewitt and Pryde. The latter authors consider that it is a form distinct from α,β -glucose, which possesses stronger chemical properties than ordinary glucose.²

Various investigators have since repeated Winter and Smith's experiments. Thus Eadie in Macleod's laboratory repeated the experiment on the blood of normal animals with exactly the same technique as Winter and Smith employed. On the whole he obtained the same results but says nothing regarding the explanation of them. Hewitt has questioned the technique which Winter and Smith used in their experiments. He says it cannot be excluded that chemical changes occur during the very complicated procedure which is necessary in order to get the glucose in a solution suitable for the polarization determination. Recently Denis and Hume repeated Winter and Smith's experiments with the original technique. They also find a diminished rotatory power in comparison

² See Paper II (Lundsgaard and Holtøll, 1925).

with the reduction power, but they maintain that corresponding values cannot be expected when the glucose is determined by two different methods neither of which in itself is specific for glucose.

EXPERIMENTAL.

A. Technique Employed.

When we wished to investigate whether we could demonstrate new-glucose in the blood of normal persons it was immediately clear that we should not use Winter and Smith's method, which is

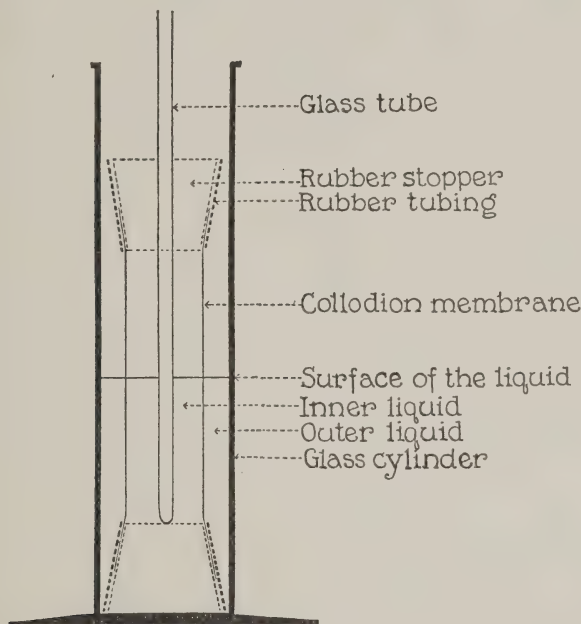


FIG. 1. Dialysis apparatus.

extremely elaborate and against which criticism has been directed. In these investigations, therefore, we determined to use the same procedure—dialysis through collodion membranes—that we had employed in our experiments *in vitro*. We will here describe the method more in detail. Fig. 1 shows the dialysis apparatus that we used in these investigations. The collodion membrane used was in the form of a cylindrical tube with a diameter of 24 mm., the bottom was closed with a solid rubber stopper to which the

collodion membrane was firmly attached by a piece of rubber tubing. After the blood, which was to be investigated, was put in the collodion membrane its other end was closed in a similar manner with a rubber stopper through which a glass tube passed down to the bottom. This glass tube served as a support for the membrane. For the dialysis the membrane was placed in a glass cylinder which was chosen of such a width that the volume of the outer liquid which was to be employed just reached as high on the collodion membrane externally as the inner liquid did internally.

The object of employing dialysis through collodion membranes was naturally to separate the crystalloids, including the glucose, from the non-diffusible colloids, *i.e.* the protein substances and the blood pigments, whose removal is necessary in order to make polarimetric readings. Collodion membranes can, however, as is known, be prepared very differently as regards the rapidity with which crystalloids diffuse through them. We have carried out a number of preliminary investigations partly to find a membrane which was best suited for these experiments and partly to ascertain whether a number of collodion membranes prepared in exactly the same manner would be constant with respect to the diffusion of different crystalloids through them. These investigations are, however, so comprehensive that a complete account of them will be given in another place. We will merely mention here that we finally chose collodion membranes which were dried in 70 per cent alcohol. In this way a membrane was obtained which prevented with certainty the passage of the protein bodies and which relatively easily allowed the glucose to pass through. Further, it was found that different membranes prepared in exactly the same manner were absolutely constant as regards the diffusion of different crystalloids. In our experiments it was our object to put the blood to be investigated in the collodion membrane and then after a suitable interval to determine the glucose in the dialysate by measurements of the rotatory and reduction powers.

Another problem, however, immediately came into prominence. Was it certain that the glucose in the blood would pass out through the membrane so that finally there would be equilibrium between the glucose content of the inner and outer liquids; or, in other words, was the glucose free in the blood or was it combined with

colloids? This problem is by no means new but has played an important rôle for a number of years and has been taken into account particularly in the different hypotheses relating to the pathogenesis of diabetes. In spite of numerous experimental investigations, however, the question was not settled when we began these experiments. It was obvious that we could not employ a technique in our investigations which required that all the glucose in the blood be dialyzed, when we did not know whether this was the case. This point had, therefore, first to be decided before we embarked upon our real investigations. The experiments with which we investigated the question as to whether the glucose occurred free in the blood or combined with colloids are, however, so extensive that they will be published elsewhere. Only the results of these experiments need be given here. We found *that the glucose both in the blood of normal persons and diabetics only occurs free.*

There was, then, nothing to prevent us from using the dialysis method in the actual experiments which we carried out with the previously employed dialysis apparatus (Fig. 1). In the experiments 40 cc. of blood were put inside the membrane and 25 cc. of NaCl solution, isotonic with the blood, outside. The glass cylinder, as mentioned was of such a width that the liquid was at the same height inside and outside. In all the experiments the dialysis was allowed to proceed at room temperature and lasted $1\frac{1}{2}$ hours. At this point it was found that equilibrium between the glucose content of the outer and inner liquids had not yet been reached, but the main portion of the glucose which can pass out into the outer liquid had gone through the membrane. We did not continue the dialysis until absolute equilibrium was reached, because the extra quantity of glucose thus obtained was small, and the extra time required might permit the new-glucose in the dialysate to change back into α, β -glucose.

In all the experiments about 2 per cent of sodium fluoride was added to the blood. Besides preventing the coagulation of the blood, glycolysis is thus avoided, which is naturally of the very greatest importance in these investigations. After $1\frac{1}{2}$ hours had passed, the dialysate was quite clear and suitable for the polarization determinations. We found with the membranes employed,

after $1\frac{1}{2}$ hours dialysis, by reduction determinations, a glucose concentration in the dialysate of about half that present in the blood at first.

Before passing on to discuss our actual experimental results we will give an account of a series of control investigations that we undertook with the object of finding out with what degree of accuracy glucose solutions of the strength used in our experiments could be determined by reduction and rotation analyses. In our experiments the glucose concentrations were about 0.05 to 0.08 per cent

TABLE I.

Control Experiments of the Accuracy of the Measurement of the Rotatory Power.

Calculated glucose concentration.	Values found by reduction determination.	Value found by rotation determination.	Specific rotatory power.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
0.10	0.100	0.100	+52.5°
0.08	0.080	0.079	+51.8°
0.06	0.060	0.060	+52.5°
0.06	0.059	0.060	+53.4°
0.05	0.050	0.048	+50.4°
0.05	0.051	0.050	+51.5°
0.05	0.050	0.050	+52.5°
0.04	0.040	0.041	+53.8°
0.04	0.040	0.041	+53.8°
0.04	0.040	0.039	+51.2°

in the dialysate. A series of glucose solutions of different concentrations was, therefore, prepared by the dilution of a stronger solution whose strength was accurately known. The glucose content of this solution was then determined both by reduction and rotation analyses. In these analyses the same technique was used as in the actual determinations of the dialysate. The reduction value was determined just as in our earlier experiments, as the mean value of four analyses by the potassium ferricyanide method. In the determination of the rotatory power, where far smaller amounts of glucose are dealt with than in the earlier experi-

ments, the mean value of forty readings was taken. In Table I all the results are recorded.

It will be seen from the table that by using this technique results are obtained which show corresponding reduction and rotation values. In harmony with this the calculated specific rotatory powers are very near $+52.5^\circ$. The greatest difference observed was 4 per cent of the value.

Our actual experiments were now performed in the following way. By venepuncture 80 cc. of blood were taken and 2 per cent of sodium fluoride was added. The blood was divided into two equal portions of 40 cc. for two collodion membranes. As outer liquid 25 cc. of 0.9 per cent NaCl solution were employed. After $1\frac{1}{2}$ hours dialysis the collodion membrane was removed and the amount of glucose in the dialysate was determined by reduction and rotation analyses as described. All the results are given in Table II.

B. Experimental Material and Conditions.

Altogether eleven normal persons, seven men and four women in ages varying from 18 to 50 years, were examined. Three of them (Nos. 8, 9, and 10 in Table II) were investigated both fasting and after the administration of 100 gm. of glucose; the other eight, all after the administration of the same amount of glucose. A higher glucose concentration in the dialysate was, of course, obtained in the latter case on account of the greater glucose concentration in the venous blood. One person was examined twice (Nos. 7 and 12). Thus fifteen determinations were made which, with a single exception (No. 4), were double determinations.

C. Experimental Results.

If we compare the values for the glucose concentration in the dialysate immediately after the dialysis was over, obtained by reduction and rotation analyses (Columns 8 and 9 in Table II), it will be observed that the value found by reduction is in all cases greater than that found by rotation. The small deviation occurring between the reduction values in the two determinations of each case are due, in addition to the negligible experimental error, to small differences in the volumes of the fluid in the dialysis appara-

TABLE II.
Investigations into the Reduction and Rotatory Powers of Glucose in the Dialysate from Blood of Normal Persons.

Case No.	Date.	Sex.	Subject.	Age.	Glucose in blood.		Glucose in dialysate immediately.		Specific rotatory power immediately.	Glucose in dialysate after 48 hrs.		Specific rotatory power after 48 hrs.	Remarks.
					(6)	(7)	Reduction.	Rotation.		Reduction.	Rotation.		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
				yrs.	per cent	per cent	per cent	per cent		per cent	per cent		
1	Sept. 19	♀	E. J.	18	0.178	0.162	0.086	0.064	+37.0°	0.086	0.082	+50.4°	1 hr. after 100 gm. glucose.
							0.079	0.061	+38.8°	0.079	0.076	+50.8°	
2	"	♂	R. N. S.	42	0.169	0.143	0.071	0.047	+34.9°	0.070	0.069	+51.4°	1 " " 100 " "
							0.073	0.044	+31.5°	0.073	0.071	+51.1°	
3	"	♂	K. K.	26	0.136	0.122	0.058	0.034	+30.7°	0.058	0.058	+52.5°	1 " " 100 " "
							0.056	0.033	+31.2°	0.056	0.054	+50.5°	
4	"	♀	A. J.	27	0.140	0.123	0.064	0.032	+26.3°	0.064	0.061	+50.1°	2 1/2 " " 100 " "
5	"	♀	A. P.	23	0.192	0.177	0.087	0.059	+35.8°	0.086	0.083	+50.7°	2 1/2 " " 100 " "
							0.084	0.054	+33.7°	0.084	0.081	+50.8°	
6	Oct. 4	♀	K. M.	24	0.157	0.140	0.076	0.036	+25.2°	0.076	0.072	+49.9°	2 1/2 " " 100 " "
							0.076	0.037	+25.9°	0.076	0.075	+51.7°	
7	"	♂	J. H. J.	39	0.151	0.133	0.069	0.023	+17.5°	0.069	0.068	+51.4°	2 1/2 " " 100 " "
							0.066	0.020	+15.9°	0.066	0.063	+50.3°	

8	Oct. 7	♂	N. C. N.	50	0.074	0.073	0.038 0.037	0.020 0.019	+26.8° +26.4°	0.038 0.037	0.037 0.037	+51.1° +52.5°	Fasting.
8 A	" 7				0.198	0.179	0.084 0.084	0.048 0.045	+29.9° +28.1°	0.084 0.084	0.082 0.081	+51.0° +50.6°	$\frac{3}{4}$ hr. after 100 gm. glucose.
9	" 9	♂	J. S.	48	0.092	0.087	0.045 0.042	0.024 0.022	+27.8° +27.5°	0.045 0.042	0.044 0.041	+51.1° +51.1°	Fasting.
9 A	" 9				0.186	0.174	0.087 0.085	0.054 0.053	+32.8° +32.7°	0.086 0.085	0.084 0.084	+51.4° +51.9°	$\frac{3}{4}$ hr. after 100 gm. glucose.
10	" 10	♂	J. V. J.	40	0.074	0.073	0.037 0.034	0.028 0.027	+39.3° +42.2°	0.037 0.034	0.037 0.033	+52.5° +51.1°	Fasting.
10 A	" 10				0.188	0.173	0.094 0.089	0.053 0.047	+29.6° +28.0°	0.094 0.089	0.091 0.087	+51.1° +51.4°	$\frac{3}{4}$ hr. after 100 gm. glucose.
11	" 11	♂	H. S.	33	0.146	0.127	0.027 0.045 0.065	0.016 0.027 0.045	+31.1° +31.5° +36.3°	0.026 0.045 0.065	0.026 0.044 0.063	+52.5° +51.1° +51.1°	$\frac{1}{2}$ hr. dialysis } $\frac{3}{4}$ hr. after 1 " " } 100 gm. 1 $\frac{1}{2}$ hrs. " } glucose.
12	" 13	♂	J. H. J.	39	0.165	0.148	0.074 0.074	0.039 0.039	+27.8° +27.8°	0.074 0.074	0.072 0.072	+51.5° +51.5°	$\frac{3}{4}$ hr. after 100 gm. glucose. Patient 7.

tus. The difference between the reduction and rotation values is most clearly seen on calculating the specific rotatory power, which is entered in Column 10. Instead of the specific rotatory power for α,β -glucose ($+52.5^\circ$), a lower rotatory power is observed in all cases. The values of the specific rotation vary between $+15.9$ and $+42.2^\circ$. It will be noticed that the double determinations of each experiment only show small deviations.

Before, however, further examining the individual results we will discuss a point which now crops up. *Is the difference found between the rotation and reduction values really due to the presence in the dialysate of a form of glucose with a lower specific rotatory power than that of α,β -glucose?* It cannot at first sight be excluded that other substances than glucose endowed with reduction and rotation powers were present in the dialysate, and that these possible substances were the cause of the altered rotatory power. This question is best decided by investigating the rotation value and from this the specific rotatory power after short and long intervals. In addition to the first rotation value, which, as mentioned, was determined immediately after dialysis, the value after a suitable interval was always read. It was then found that the rotation value slowly rose and became constant after 48 hours. The course of the mutarotation in these 48 hours will be discussed later in this paper. At this time the reduction values of the dialysate were again determined and are recorded in Columns 11 and 12 of Table II, and the specific rotatory power calculated from these determinations is given in Column 13. If now we first compare the reduction value for glucose in the dialysate immediately and after 48 hours, we find absolutely identical values. We can therefore assert that no loss of glucose has taken place in this interval. But it will be noticed that the rotatory power in all cases is far higher after 48 hours than it is immediately, so that the rotation values which are now observed are very nearly equal to the corresponding reduction values. This is also seen on calculating the specific rotatory power which in all cases is very near $+52.5^\circ$. This must signify that the reducing bodies in the dialysate after 48 hours are α,β -glucose. The reason that the true value for α,β -glucose ($+52.5^\circ$) is not quite reached, for in the great majority of cases the specific rotatory power is rather lower, must be that other reducing bodies than glucose are present in the dialysate; namely,

uric acid and creatinine. As will be seen from the table by comparing the reduction and rotation values in the dialysate after 48 hours, the reduction due to creatinine and uric acid can only be slight. If we take the mean of all the experiments it will be found that the reduction value is 2.5 per cent higher than the rotation value. That is to say, only 2.5 per cent of the reduction value of the dialysate is due to other reducing bodies than glucose. These substances are probably uric acid and creatinine. We obtained a confirmation of this in another way. In five of the patients investigated we determined the content of the venous blood in creatinine and uric acid as well as glucose. These two substances were determined by Folin and Wu's method. In Table III the mean values are recorded for the amounts of glucose, uric acid, and creatinine in the blood of the five patients.

TABLE III.

Mean Values of the Glucose, Creatinine, and Uric Acid Contents of the Blood of Five Patients after the Administration of 100 Gm. of Glucose.

In 100 cc. blood we found:

	mg.
Glucose.....	158 0
Creatinine.....	3 5
Uric acid.....	2.8

It will be seen from the table that a total of 6.3 mg. of uric acid and creatinine correspond to 158 mg. of glucose. The amounts of uric acid and creatinine have, therefore, constituted 4 per cent of the amount of glucose in these five cases. This, however, does not mean that the reduction power of these substances is 4 per cent of glucose, because the reduction power of these three substances in the presence of potassium ferricyanide is different.

We have studied the question in a series of investigations. We found that the reduction power of creatinine and uric acid in the presence of potassium ferricyanide cannot be stated as a constant percentage of the reduction of the glucose. We found that the reduction power of creatinine and uric acid with respect to that of the glucose is dependent upon the active amount of creatinine and uric acid. It appeared from the analyses that the reduction of potassium ferricyanide was greatest, compared with that of the

glucose, with the smallest amounts. For the quantities found in blood we can without any serious error put the reduction power at 60 per cent of that of the glucose. In the cases we investigated the reduction of the uric acid and creatinine on the blood would constitute 2.4 per cent of that of the glucose.

However, we investigated the reduction in the dialysate³ and not in the blood, so that it was necessary to discover the rate at which the three substances under identical conditions passed through the collodion membrane. In some orientating experiments we found that there was only a slight difference in the rate of diffusion through the same membrane for glucose, uric acid, and creatinine; the last two substances, however, in accordance with their rather smaller molecules than those of glucose, passed through the collodion with a rapidity which was slightly greater than that of glucose. The reduction of these two substances in the dialysate must, therefore, be slightly greater than 2.4 per cent of that of the glucose, a value which agrees very well with the difference of 2.5 per cent which we found between the reduction and the rotation values in the dialysate after 48 hours. There is thus no doubt that the glucose in the dialysate after 48 hours is α,β -glucose with the specific rotatory power of $+52.5^\circ$.

We will now return to the results which we found on immediate investigation of the dialysate. It is now proved that the rotation values are the true rotatory values for the glucose in the dialysate and that the reduction values are only subject to such a small error that we are warranted in neglecting it. *Consequently the values given in Column 10 must represent accurately the specific rotatory power of the glucose in the dialysate immediately after the dialysis is finished.* It is thus shown that the glucose in the dialysate, at any rate partly, must be present in a form that is distinct from α,β -glucose.

To determine whether this form was identical with the new-glucose demonstrated by us in the experiments *in vitro*, we compared the mutarotation of these substances. As previously mentioned, the rotation values in the dialysate were measured in all cases at suitable intervals until constant values were reached. These are

³ The reason that we did not determine the amounts of creatinine and uric acid directly in the dialysate which would naturally have been easier, was that Folin and Wu's method proved not to be suitable for this.

entered in Column 12, Table II. As the mutarotation was found to follow the same course in each case the results from only two of the cases will be given here, as examples. In Fig. 2 and in Table IV the course of the mutarotation is given for Case 12 of Table II. It will be seen that the process proceeds most rapidly in the first 24 hours and does not come to an end before 48 hours have passed, from which time the rotation value remains constant. Exactly similar results are seen in Fig. 3 and in Table V where the mutarotation of the glucose in the dialysate of Case 6 of Table II is recorded.

In order to compare the mutarotation of the form prepared *in vitro* by the addition of insulin and muscle tissue to glucose (for which we have proposed the provisional name, new-glucose) and the form of glucose present in the blood of normal persons, we have recorded in Fig. 4 and Table VI the course of the mutarotation of: (1) the glucose in the dialysate from normal human blood; (2) new-glucose prepared *in vitro* from α, β -glucose by the addition of insulin and fresh muscle tissues; and (3) chemically prepared β -glucose.

In all cases the glucose was kept under identical conditions dissolved in a neutral liquid at 18°C.

The investigation of new-glucose prepared *in vitro* is taken from Paper I, Table VI. A case was chosen in which the specific rotatory power at the first examination had a value which corresponded with those found for the glucose in the dialysate from blood.

It is clear from the results of the experiments that the form of glucose (new-glucose), which we prepared by letting insulin and fresh muscle tissue act upon α, β -glucose, shows a mutarotation which is widely different from that of β -glucose, but in every case coincides with the mutarotation of the form of glucose found in the dialysate from normal human blood. We are therefore justified in drawing the following conclusion from our results: *new-glucose can always be demonstrated in normal human blood.*

If we now study the individual results a little closer we shall see, on inspecting the immediate values of the specific rotatory power in the dialysate (Column 10, Table II), that these values are rather different in the cases investigated, for they vary from +15.9° to +42.2°. But the values of the double analyses do not diverge more than the experimental error would account for.

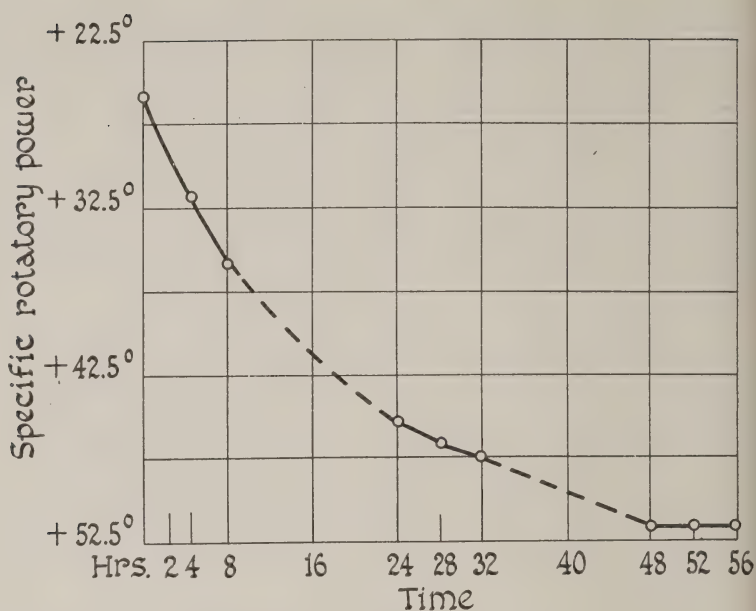


FIG. 2. The mutarotation of the new-glucose in the dialysate. Case 12, Table II.

TABLE IV.

Mutarotation of the Glucose in the Dialysate from Blood.

Case 12, Table II. See also Fig. 2.

Time at end of dialysis.	Glucose rotation value.	Specific rotatory power.
<i>hrs.</i>	<i>per cent</i>	
0	0.039	+27.8°
4	0.047	+33.1°
8	0.052	+36.7°
24	0.066	+46.6°
28	0.067	+47.5°
32	0.068	+48.4°
48	0.072	+51.5°
52	0.072	+51.5°
56	0.072	+51.5°

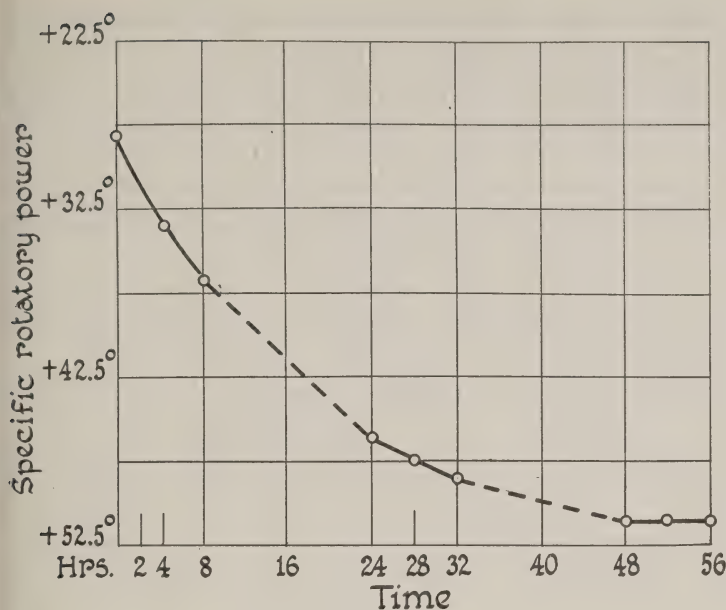


FIG. 3. The mutarotation of the glucose in the dialysate from the blood. Case 6, Table II.

TABLE V.

Mutarotation of the Glucose in the Dialysate.

Case 6, Table II. See Fig. 3.

Time at end of dialysis.	Glucose rotation value.	Specific rotatory power.
<i>hrs.</i>	<i>per cent</i>	
0	0.037	+25.9°
4	0.046	+32.1°
8	0.052	+36.0°
24	0.065	+45.3°
28	0.068	+46.8°
32	0.069	+47.5°
48	0.075	+51.7°
52	0.075	+51.7°
56	0.075	+51.7°

As we know that new-glucose has a lower specific rotatory power than α, β -glucose, the specific rotatory power of the glucose in the dialysate will be lower the higher the concentration of new-glucose. It follows from this that the amount of new-glucose varies in the different cases.

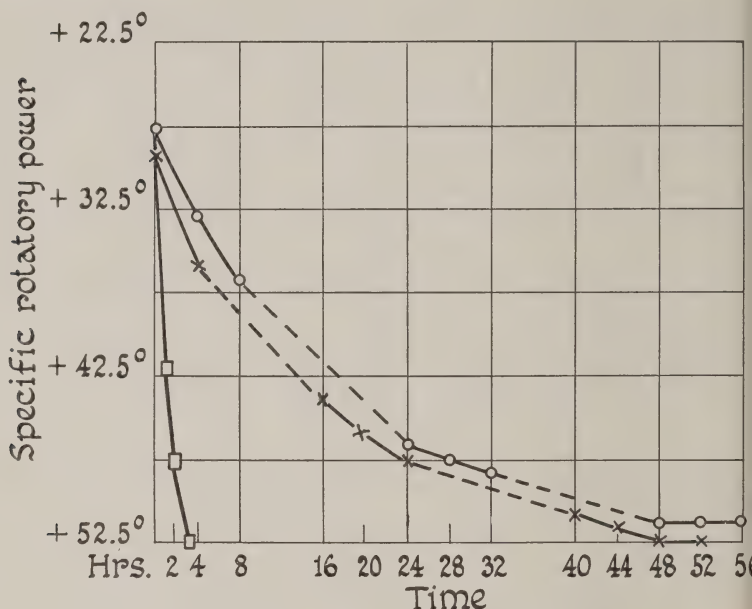


FIG. 4. Comparative curves of the course of the mutarotation of the glucose in the dialysate from blood, new-glucose prepared *in vitro*, and β -glucose. In all the experiments the temperature was 18°C. and the reaction neutral.

- o-----o Glucose in dialysate from blood.
- x-----x New-glucose prepared *in vitro*.
- β -Glucose.

If we first examine the three persons who were investigated while fasting, we find the following values for the specific rotation: Case 8, +26.8° and +26.4°; Case 9, +27.8° and +27.5°; and Case 10, +39.3° and +42.2°.

New-glucose is, therefore, also present in the blood of normal fasting persons and at any rate, as regards the first two, in as great a concentration as after the administration of glucose. All the

other determinations, as mentioned, were made after giving 100 gm. of glucose—in three cases 1 hour afterwards, and in the other nine cases only $\frac{3}{4}$ hour afterwards. New-glucose was again demonstrated in the blood in all cases, but in a different concentration. The greatest quantity of new-glucose was found in Case 7 where the specific rotatory power was $+17.5^\circ$ and $+15.9^\circ$. There is a special reason for drawing attention to this result because these values are below the lowest specific rotatory value which could be

TABLE VI.

Mutarotation of the Glucose in the Dialysate from Blood, New-Glucose, and β -Glucose.

Time. <i>hrs.</i>	Specific rotatory power.		
	Glucose in dialysate. Case 12, Table II.	New-glucose.	β -Glucose.
0	$+27.8^\circ$	$+29.6^\circ$	
$\frac{1}{2}$ 0			$+24.5^\circ$
1			$+42.2^\circ$
2			$+48.7^\circ$
3			$+52.5^\circ$
4	$+33.1^\circ$	$+35.9^\circ$	
8	$+36.7^\circ$		
16		$+43.8^\circ$	
20		$+45.7^\circ$	
24	$+46.6^\circ$	$+47.5^\circ$	
28	$+47.5^\circ$		
32	$+48.7^\circ$		
40		$+50.2^\circ$	
44		$+51.8^\circ$	
48	$+51.5^\circ$	$+52.5^\circ$	
52	$+51.5^\circ$	$+52.5^\circ$	
56	$+51.5^\circ$		

found even in all the most favorable cases if the glucose in the dialysate was β -glucose; namely, $+19^\circ$. They constitute direct experimental proof that the glucose in the dialysate is not β -glucose. This also follows, moreover, from the widely different mutarotation course, as was shown in Fig. 4 and in Table VI. This patient was examined again 3 days later, but at this examination the specific rotatory power was found to be $+27.8^\circ$ in both experiments. This informs us that the quantity of new-glucose in

the blood of the same individual under otherwise uniform conditions is variable. We are unable to draw any conclusions from these experiments regarding the cause of this varying concentration of new-glucose in the blood. As stated, we first investigated the glucose in the dialysate $1\frac{1}{2}$ hours after the blood was drawn. As new-glucose reverts to α, β -glucose on standing the question arises whether new-glucose is not present in a still greater concentration in the blood itself than we find after $1\frac{1}{2}$ hours dialysis. We have attempted to throw some light on this problem in our investigation of Case 11. In this case 120 cc. of blood were taken and 40 cc. were put in each of three collodion membranes. The dialysate was examined at different times in the different experiments; namely, after intervals of $\frac{1}{2}$, 1, and $1\frac{1}{2}$ hours. The following specific rotatory values were found (see Table II): $\frac{1}{2}$ hour, $+ 31.1^\circ$; 1 hour, $+ 31.5^\circ$; and $1\frac{1}{2}$ hours, $+ 36.3^\circ$.

The experiment shows that there is no great change in the specific rotatory power during the dialysis, which means that the concentration of new-glucose is not diminished to any great extent. This agrees, moreover, with what we know about the mutarotation of new-glucose; namely, that at room temperature it takes rather a long time (48 hours).

As will be remembered from our first article of this series, we showed that in the dialysate from a mixture of glucose, fresh blood, and insulin no difference between the reduction and rotation values of glucose in excess of the experimental error could be detected. As we now know that the added blood must have contained new-glucose it might be asked why the latter did not show in the results. The reason is that the amount of new-glucose in the dialysate in these experiments was so small in comparison with the amount of unchanged α, β -glucose that the influence of the new-glucose on the results was within the experimental error.

As will be seen from Table II we have also determined the concentration of glucose in the venous blood and in the cutaneous blood (arterial blood) in all the cases. The results are given in Columns 6 and 7 of Table II. As will be observed, a well marked difference between the sugar content of the arterial and venous blood after taking glucose is found in every case. This point will be further investigated in a later work.

DISCUSSION.

A. How is New-Glucose Formed?

Where does the new-glucose demonstrated in normal human blood come from? To decide this question we can make use of our earlier experiments *in vitro*. It will be remembered that we showed that new-glucose is formed by the action of muscle tissue and insulin on α, β -glucose, while insulin alone or insulin and blood had no such effect. It is, therefore, natural to assume that the same two factors, insulin and a substance or principle present in muscle tissue, are necessary for the production of this body in the organism. Both these components are always present in the normal person.

B. How Does the Formation of New-Glucose Take Place?

We do not yet know how this body is produced. We can imagine that the action upon α, β -glucose occurs in the blood stream, the necessary substance diffusing into the latter from the muscles and in conjunction with insulin transforming the α, β -glucose into new-glucose. But it is also possible that the α, β -glucose absorbed from the intestinal canal through the capillary walls diffuses out into the muscle tissue where the transformation then takes place.

C. What Is the Subsequent Fate of New-Glucose in the Organism?

We know nothing certain about the subsequent fate of new-glucose. In the earlier experiments *in vitro* no further transformation occurred, but the new-glucose gradually reverted to α, β -glucose. *In vivo*, however, its effect is certainly different. Since we know that this substance is formed from ordinary glucose by the action of insulin and muscle tissue, and since we have now shown that it can be always detected in normal human blood there are very good grounds for believing that it is a link in the chain of transformation, and probably the first, of glucose in the normal combustion of carbohydrate, a view we have already put forward in our first work.

More light would, however, undoubtedly be thrown on this problem by investigating the conditions in pathological carbo-

hydrate metabolism; that is to say, in patients with diabetes mellitus. Investigations into the conditions in these patients are already under way and we hope shortly to be able to report the results.

SUMMARY.

1. A method is described for determining the specific rotatory power of glucose in the blood.

2. In fifteen experiments on eleven normal persons a lower specific rotatory power than that of α, β -glucose was found in all cases. This shows that a form of glucose is present in the dialysate with a lower specific rotatory power. The values measured varied between $+15.9^\circ$ and $+42.2^\circ$.

3. The form of glucose thus demonstrated reverts to α, β -glucose. On studying the course of the mutarotation a complete agreement was found with that previously found for new-glucose.

4. It must, therefore, be taken as proved that new-glucose can always be detected in the blood of normal persons both while fasting and also after taking glucose.

5. This result, taken together with the previously demonstrated mode of production of new-glucose, supports the view that new-glucose is the first link in the chain of the transformation of glucose in normal carbohydrate metabolism.

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STUDIES IN CARBOHYDRATE METABOLISM.

V. INVESTIGATIONS INTO THE NATURE OF THE GLUCOSE IN THE BLOOD OF PATIENTS WITH DIABETES MELLITUS AND OF PATIENTS WITH BENIGN GLYCOSURIA.

BY CHRISTEN LUNDGAARD AND SVEND AAGE HOLBØLL.

From Medical Clinic A, University of Copenhagen, Copenhagen, Denmark.)

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INTRODUCTION.

In an earlier article,¹ we showed that there is a form of glucose in the blood of normal persons which is different from ordinary glucose (α,β -glucose). This form was demonstrated in all the cases examined both when the individuals were fasting and after they had taken glucose. The occurrence of this form of glucose in the blood could be detected by means of the specific rotatory power which was lower than the value for ordinary α,β -glucose. We demonstrated this form of glucose in the dialysate from blood, and found that it reverted to α,β -glucose in the dialysate. This reversible process was further studied and it was found that the mutarotation of the glucose in the dialysate from the blood of normal persons was identical in every case with the mutarotation of that form of glucose we previously had prepared by adding insulin and fresh muscle tissue to a solution of α,β -glucose. We, therefore, felt justified in drawing the conclusion that these forms of glucose detected in different ways were identical. We have proposed to call this form of glucose, *new-glucose*, until further information about its chemical structure has been obtained.

Our knowledge of the production of this form of glucose *in vivo* coupled with our demonstration of its constant occurrence in the blood of normal persons permitted us to draw the con-

¹ Lundsgaard and Holbøll (1924, 1925, *a* and *b*).

clusion: *new-glucose is a link, and probably the first, in the chain of transformation in normal carbohydrate combustion.*

The question then arose, what was its relation to the pathological carbohydrate transformation in patients with diabetes mellitus? In accordance with our present knowledge of new-glucose these investigations could be carried out in the same way as the determinations were in normal persons; namely, by estimating the specific rotatory power of glucose in the blood of patients with diabetes mellitus.

Earlier Investigations into the Specific Rotatory Power of Glucose in the Blood of Patients with Diabetes Mellitus.

Such investigations have been previously undertaken by Winter and Smith, with exactly the same technique as the one employed in their investigations into the nature of glucose in normal persons, as we mentioned in our previous article. In their first contribution they found as the result of five examinations of patients suffering from diabetes that the reduction and rotation values of the glucose were equally great. In a short paper soon afterwards they state, without giving any experimental results, that the rotation value of glucose in the blood of diabetics was usually rather greater than the reduction value which they considered was due to the presence of other kinds of sugar, perhaps disaccharides or some other complex form. While Winter and Smith's investigations in normal persons, as already mentioned, have been repeated several times, this has not been the case with the investigations referred to in patients with diabetes mellitus.

EXPERIMENTAL.

A. Technique Employed.

In these investigations we used exactly the same method and procedure as in our experiments with normal persons, which are described in Paper III,² to which paper the reader is therefore referred. The experiments were made by putting the blood in the collodion membrane through which the glucose dialyzed. The glucose in the dialysate was then determined both by

² Lundsgaard and Holbøll (1925, b).

reduction and its rotation powers and from these determinations the specific rotatory power was calculated. As described in our preceding work, it was necessary, before carrying out our experiments with this technique, to be certain that the glucose, in diabetic blood also, was only present in a form which would dialyze. We, therefore, made a series of preliminary experiments which will be published elsewhere and which led to the result: *glucose occurs exclusively in a free state both in normal and diabetic blood.*

The method used in the actual experiments was, as stated, absolutely the same as that previously employed. By venepuncture 80 cc. of blood were taken which, after the addition of 1 per cent of sodium fluoride to prevent glycolysis, was divided into equal parts of 40 cc. and put in two collodion membranes. As external liquid 25 cc. of physiological salt solution were used. The period of dialysis was $1\frac{1}{2}$ hours and dialysis took place at room temperature. The reduction power was determined, as previously mentioned, as the mean of four analyses by the potassium cyanide method, and the rotatory power as the mean of forty readings.

B. Experimental Material and Conditions.

First of all, ten patients suffering from diabetes mellitus were examined. When the investigation was made the patients had not been treated with insulin. All the results are recorded in Table I.

As will appear from Table I, examinations were made on ten patients, one being examined twice (Nos. 9 and 11). All the examinations, with one exception, were double determinations. Of the patients investigated, five were men and five women. The ages varied between 16 and 60 years. The patients were all examined at the same time of day; namely, 1 hour after they had had breakfast. In addition to the determination in the venous blood, a sample of cutaneous blood (arterial blood) was taken from the ear for the determination of the glucose concentration. All the patients examined had severe or moderately severe diabetes mellitus. The patients were investigated at different times during their stay in the hospital, a few shortly after admission, and others after a longer or shorter period of treatment in the

TABLE I.
Specific Rotatory Power of Glucose in the Dialysate from the Blood of Patients with Diabetes Mellitus.

Case No.	Date.	Sex.	Patient.	Age.	Glucose in ear blood.	Glucose in venous blood.	Glucose in dialysate immediately.		Specific rotatory power immediately.	Glucose in dialysate after 48 hrs.		Specific rotatory power after 48 hrs.	Remarks.
							Reduction.	Rotation.		Reduction.	Rotation.		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
					per cent	per cent	per cent	per cent		per cent	per cent		
1	Sept. 23	♀	A. K. L.	60	0.210	0.210	0.119	0.114	+50.2°	0.119	0.115	+51.1°	
							0.116	0.113	+50.6°	0.116	0.115	+52.1°	
2	" 24	♂	C. L. J.	42	0.163	0.162	0.086	0.086	+52.5°	0.085	0.084	+51.9°	
							0.082	0.080	+51.5°	0.082	0.081	+52.0°	
3	" 26	♂	K. N.	37	0.099	0.092	0.047	0.040	+44.7°	0.047	0.046	+51.1°	Examined shortly before discharge.
							0.047	0.040	+44.7°	0.047	0.046	+51.1°	
4	" 30	♀	K. P.	33	0.280	0.279	0.134	0.129	+50.6°	0.134	0.132	+51.7°	
							0.128	0.124	+50.9°	0.127	0.123	+51.0°	
5	" 30	♀	K. P.	21	0.360	0.360	0.174	0.169	+51.0°	0.173	0.165	+50.1°	
							0.186	0.182	+51.4°	0.186	0.182	+51.4°	
6	Oct. 6	♂	V. P.	23	0.235	0.236	0.128	0.124	+50.8°	0.128	0.124	+50.8°	
7	" 8	♂	S.	27	0.390	0.390	0.170	0.167	+51.5°	0.170	0.168	+51.7°	
							0.214	0.210	+51.4°	0.214	0.212	+51.9°	
8	" 9	♂	H. A.	56	0.295	0.294	0.153	0.150	+51.4°	0.153	0.151	+51.7°	

9	13	♀	J. H.	16	0.630	0.306	0.293	+50.3° +51.4°	0.305 0.283	0.292 0.278	+50.3° +51.6°	Incipient coma.
10	"	♀	B.	54	0.336	0.174 0.160	0.171 0.157	+51.4° +51.6°	0.174 0.160	0.171 0.156	+51.4° +51.2°	
11	Nov. 10	♀	J. H.	16	0.182	0.094 0.094	0.061 0.059	+34.2° +33.1°	0.094 0.093	0.089 0.090	+50.0° +51.0°	Patient 9. Examined af- ter 1 mo. treatment.

hospital. This will be further discussed later. Five of the diabetic patients, namely Nos. 1, 2, 4, 6, and 7 in Table I, were examined later after the administration of insulin. The examination in all cases took place 2 hours after the insulin injection. Further, two patients were examined who had glycosuria of a benign type.

C. Experimental Results.

1. *Diabetics without Insulin.*—In Table I where all the results of the investigations of diabetics without insulin treatment are recorded, the reduction and rotation values for glucose in the dialysate immediately after dialysis are given in Columns 8 and 9. If we first consider the reduction values it will be noticed that in the great majority of cases we are dealing with greater concentrations than were found in the dialysate from normal human blood, which is in agreement with the higher glucose concentration in the blood of diabetic patients. On comparing the values for the glucose concentration calculated from the reduction and rotation values, it will be observed that in nine out of the eleven cases they very nearly agreed with one another; in two cases (Nos. 3 and 11) only is there a decided difference.

If we now examine more closely the nine cases where the reduction and rotation values are about the same, we can best judge the results obtained by considering the values for the specific rotation which are entered in Column 10, and which for these cases show values varying between $+50.2^\circ$ and $+52.5^\circ$. We can, therefore, conclude that the glucose in the dialysate occurs α, β -glucose. The reason that the theoretical specific rotatory power for α, β -glucose ($+52.5^\circ$) is not reached and the values found are a little lower, must be due to the same conditions which obtained after the mutarotation in the dialysate from normal persons' blood was at an end; namely, the presence of small quantities of uric acid and creatinine. This is confirmed also by investigating the rotation value afterwards. This was determined at suitable intervals for 48 hours. In these nine cases no change in the rotation value in excess of the experimental error was ever observed. The reduction value was also determined again after 48 hours. These values, together with the value of the simultaneously measured rotatory power and the

specific rotatory power calculated from it, are entered in Columns 11, 12, and 13 in Table I. It appears, therefore, that the values of the reduction power entirely agree with the value found immediately after dialysis. This proves that no breaking down of the glucose has occurred. Next it is observed that in all the nine experiments values for the specific rotatory power were obtained which within the experimental error are identical with those previously determined.

We may, therefore, draw the conclusion that the rotation value in the dialysate is only due to glucose, and that the reduction value for the greater part is also due to glucose and only a small percentage of it is accounted for by uric acid and creatinine. This result was unexpected because in the blood of diabetic patients another dialyzable substance besides glucose, which affects polarized light, namely β -hydroxybutyric acid, can undoubtedly occur.

The majority of the nine patients examined certainly did not excrete acetone in the urine at the time the investigation took place, but in one or two there was distinct though not very marked acetonuria. We can, therefore, only say that the amount of β -hydroxybutyric acid which may have been present in these patients' blood, could not have had any measurable influence on the rotatory power of the dialysate, without, however, at present being able to decide why this was not the case.

From our investigations on the nine patients we can assert: *After 1½ hours' dialysis at room temperature of blood from patients with diabetes mellitus the glucose in the dialysate occurs as α, β -glucose.* Naturally we cannot conclude from this that the glucose in the blood was only α, β -glucose, but we can truly say that, using the same technique which in all cases showed the presence of new-glucose in the dialysate from normal human blood, we are unable to demonstrate this substance in the blood of nine patients with diabetes mellitus.

In Table I, however, there are two patients which deviate from his result. In No. 3 the specific rotatory power of the glucose in the dialysate immediately was $+44.7^\circ$, and after 48 hours, $+51.1^\circ$. In No. 11 immediately after dialysis it was $+34.2^\circ$ and $+33.1^\circ$, but after 48 hours, $+50.0^\circ$ and $+51.0^\circ$. Besides the values measured immediately and after 48 hours,

the rotation value and therefrom the specific rotatory power were always measured as previously. It was then found that constant values were only reached after 48 hours and that the mutarotation also completely coincided with that of the glucose in the dialysate from normal human blood. That the lower values of the rotatory power in these two cases might be due to the presence of β -hydroxybutyric acid is excluded because these two patients did not excrete acetone and diacetic acid in the urine at the time the examination took place.

We are, therefore, justified in assuming that in the blood of these two patients the same form of glucose as we found in normal human blood occurs; namely, new-glucose. It was, therefore, worth while examining these patients further.

In the case of Patient 3 the investigation was made after the patient had been on strict dietetic treatment for $1\frac{1}{2}$ months and immediately before discharge from the hospital. The low glucose concentration in the blood (0.099 per cent) 1 hour after breakfast shows most clearly that the patient was improving when the examination was made. The specific rotatory value in the concordant double analyses is lower than that measured after 48 hours and is considerably in excess of the experimental error. The values found, however, are a little higher than those found in any of our normal persons, indicating that the concentration of new-glucose is not so high as it was in the normal person investigated. This was, however, the case in Patient 11 where the values found for the glucose in the dialysate immediately after dialysis correspond absolutely with what is usually obtained in normal persons. This case is specially interesting because the patient was examined twice; namely, on admission (No. 9) and after 1 month's treatment (No. 11). On admission the patient was in the early stage of coma. The blood sugar content was very high (0.63 per cent). At this time, as will be seen from Table I, there was no difference between the rotation and reduction values of the glucose in the dialysate. The patient was treated with insulin and a strict diet and improved very quickly so that the dose of insulin could be steadily diminished and finally discontinued. The last examination was made when the patient had received no insulin for 3 days. At this time the conditions were found, as referred to, which justified us in assuming the presence of new-glucose.

These last investigations show us that there are cases in which new-glucose can be demonstrated in the blood of diabetics who have passed through a course of rational treatment and are considerably improved clinically. It would, however, be interesting to follow a number of these patients during their treatment. It is quite clear, however, that this cannot be done with the technique hitherto employed, which requires 80 cc. of blood for the two determinations. We are, however, elaborating a method by which the investigations can be carried out with

TABLE II.

Review of Some of the Clinical Data of the Patients Investigated.

Case No.	Length of time symptoms were noticed before admission.	Length of time after admission that the investigation in Table I took place.	Fasting blood sugar at time of examination.	State of urine at time of examination.		
				Glucose.	Diacetic acid.	Acetone.
			<i>per cent</i>	<i>per cent</i>		
1	2 yrs.	4 days	0.242	+7.5	—	+
2	1 $\frac{1}{4}$ "	1 mo.	0.156	—	—	—
3	1 mo.	40 days	0.079	—	—	—
4	2 mos.	18 "	0.294	+1.5	—	—
5	2 "	2 $\frac{1}{2}$ mos.	0.294	+0.5	—	—
6	$\frac{1}{2}$ hr.	1 day.	0.211	+3	+	+
7	8 yrs.	1 "	0.288	+3	—	—
8	2 $\frac{1}{2}$ "	2 days.	0.240	+4	—	—
9	1 wk.	0 "	0.355	+10	—	+
10	2 mos.	2 "	0.216	+7	+	+

smaller quantity of blood and we hope to throw further light on certain points by this means.

The results so far show that while new-glucose can always be detected in the dialysate from normal human blood, it was only demonstrated in two cases out of eleven with the same technique, and these two patients were clinically considerably improved when the examination took place.

We need not go into further detail as regards the clinical picture of each of the diabetic patients investigated, but merely refer to Table II in which is entered how long each patient had noticed symptoms before admission to the hospital, and how long

after admission the investigation recorded in Table I was made. Information is also given concerning the patients' condition at the time the investigation was carried out; namely, the fasting blood sugar and the content of glucose, acetone, and diacetic acid in urine.

Glucose Concentration in Ear and Venous Blood.

In Columns 6 and 7 of Table I are given, as previously mentioned, the simultaneously determined glucose concentrations in the venous blood and the cutaneous blood from the ear (arterial blood). It will be seen that in nine of the eleven cases in the table there was no demonstrable difference between the glucose concentration in arterial and venous blood in spite of the fact that the investigation was made after food was taken, definitely proving that no detectable quantity of glucose was removed

TABLE III.

Blood Sugar Concentration in the Cutaneous Blood (Arterial Blood) and Venous Blood in Two of the Patients Investigated.

Case No.	Ear blood.	Venous blood.	Difference.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
3	0.099	0.092	0.007
11	0.204	0.182	0.022

from the blood stream during its passage through the capillary system. These nine cases were those in which new-glucose was not detected in the dialysate from the blood. This result is absolutely different from what was found in normal persons where a well marked difference between the glucose content of the arterial and venous blood was found in all cases after taking carbohydrate, which shows that some of the glucose was removed from the blood stream during its passage through the capillary system.

In two of the diabetic patients a difference between the glucose content of the arterial and venous blood was found, as appears from Table III.

Table III shows that these patients are able to remove some of the glucose in the capillary system. It will be remembered that it was in these two patients that we demonstrated new-glucose in the dialysate from the blood.

We can, therefore, say for the present: in those patients investigated in whom some of the glucose was removed in its passage through the capillary system, namely all the normal persons examined and the diabetics Nos. 3 and 9, we were able to demonstrate new-glucose in the dialysate from the blood. In the nine diabetics in whom no removal of glucose in its passage through the capillary system was found, new-glucose could not be demonstrated. This will be further discussed later.

2. *Patients with Diabetes Mellitus after the Administration of Insulin.*—We have now shown that the form of glucose which we have proposed to call new-glucose for the present, and which is always found in the blood of normal persons, cannot be detected in the majority (nine out of eleven) of patients with severe attacks of diabetes. We found in fact that in these nine cases, to which all the untreated belong, the glucose in the dialysate, which was investigated with exactly the same technique as that hitherto employed, was entirely α, β -glucose.

The question, therefore, arose as to what was the condition in diabetic patients after insulin. All the results are entered in Table IV. Altogether five patients were investigated and, as mentioned, those were chosen who had been investigated before they underwent treatment with insulin; namely, Nos. 1, 2, 4, 6, and 7 in Table I. At the first examination of the blood of these patients no difference between the reduction and rotation values was found, which means that new-glucose could not be demonstrated.

Between the first examination of these patients which is recorded in Table I, and the last, after the administration of insulin, a variable period—as can be seen from the tables—elapsed; namely, 1, 4, 11, 14, and 32 days in the five cases. It may, however, be remarked that even in the patients where there had been a long interval between the two examinations, their clinical conditions were unchanged. As will be observed from the table, four out of the five examinations were double determinations. Otherwise the results are recorded in the usual way. From the results of the reduction and rotatory powers of the dialysate immediately after dialysis (Columns 8 and 9) it appears that in all cases there is a distinct difference, the rotation values being less than the corresponding reduction values. This

TABLE IV.
Specific Rotatory Power of the Glucose in the Dialysate from the Blood of Patients with Diabetes Mellitus 2 Hours after the Administration of Insulin.

Case No.	Date.	Sex.	Patient.	Age.	Glucose in ear blood.		Glucose in venous blood.		Glucose in dialysate immediately.		Specific rotatory power immediately.	Glucose in dialysate after 48 hrs.		Specific rotatory power after 48 hrs.	Remarks.
					(6)	per cent	(7)	per cent	Reduction.	Rotation.		Reduction.	Rotation.		
(1)	(2)	(3)	(4)	(5)	(6)	per cent	(7)	per cent	(8)	(9)	(10)	(11)	(12)	(13)	(14)
1	Oct. 4	♂	C. L. J.	42 yrs.	0.188	0.170	0.084	0.056	0.084	0.056	+35.3°	0.084	0.082	+51.4°	Patient 2. 14 units insulin.
2	" 7	♂	V. P.	23	0.459	0.430	0.231	0.169	0.231	0.169	+38.4°	0.231	0.213	+48.5°	Patient 6. 12 units insulin.
3	Sept. 27	♀	A. K. L.	60	0.241	0.203	0.222	0.162	0.222	0.162	+38.4°	0.222	0.201	+47.7°	Patient 1. 10 units insulin.
4	Oct. 14	♀	K. H. H.	33	0.087	0.074	0.037	0.021	0.037	0.021	+27.3°	0.035	0.035	+49.8°	Patient 4. 8 units insulin.
5	Nov. 10	♂	S.	27	0.298	0.294	0.142	0.113	0.142	0.113	+41.9°	0.141	0.139	+51.5°	Patient 7. 18 units insulin.
							0.142	0.119	0.142	0.119	+44.0°	0.142	0.140	+51.7°	

shows most clearly when the specific rotatory value is calculated, which value is entered in Column 10. The values obtained lie between $+26.5^\circ$ and $+44.0^\circ$. These rotation values were followed in the usual manner until the rotation became constant. This did not occur until 48 hours had elapsed in all the cases. At this time the reduction value was again examined as usual, and as appears from the table (Column 11) it was identical with the first determination. In every case the specific rotatory power approximated to the value for α,β -glucose: $+52.5^\circ$. The rather lower value which is constantly found is due, as before, to the content of uric acid and creatinine.

In Fig. 1 and Table V the mutarotation of the glucose in one of the dialysates is given. The others, as stated, show exactly the same course. If we compare the values in Fig. 1 and Table V with the values which we previously found for the glucose in the dialysate from the blood of normal persons and with the form of glucose prepared *in vitro*, it will be found that the mutarotation proceeds in exactly the same manner.

The mutarotation alone shows that the lower values of the specific rotation observed at the first examination again in these experiments cannot have any connection with the presence of β -hydroxybutyric acid in the blood, but it may be added that these patients had no acidosis when the investigation was carried out. The result of these experiments is, therefore, as follows: *in five patients suffering from diabetes mellitus in whom new-glucose could not be detected in the blood before insulin treatment, this substance was found in all cases 2 hours after an insulin injection.*

If we further examine the figures for the specific rotatory power immediately after dialysis we shall find that in two cases, Nos. 3 and 5, the values are a little higher than those which were found in normal persons, and in one case, No. 2, the value coincided with the uppermost limit of what was found in normal persons. This must indicate that the relation between the amount of new-glucose and that of α,β -glucose is less in these cases than in the investigations which were undertaken on normal persons. This is, however, only a natural consequence of the fact that the entire glucose concentration of the blood is greater than in normal persons, which is specially pronounced in the three patients with the highest specific rotatory powers.

In this series of investigations, just as in those described earlier determinations were undertaken of the glucose concentration in the venous blood as well as in the cutaneous blood from the

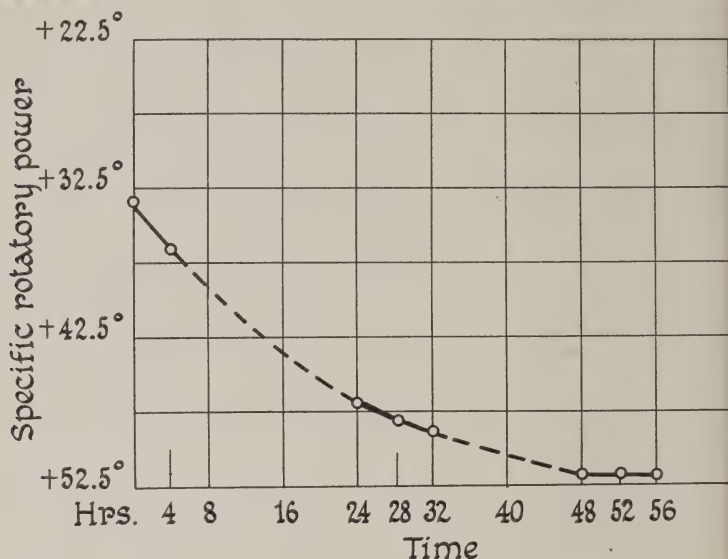


FIG. 1. The mutarotation of glucose in the dialysate. Case 1, Table II.

TABLE V.

Mutarotation of the Glucose in the Dialysate from Table IV, No. 1 (see Fig. 1)

Time.	Rotation value of glucose.	Specific rotatory power.
<i>hrs.</i>	<i>per cent</i>	
0	0.049	+33.4°
4	0.054	+36.5°
24	0.070	+47.2°
28	0.071	+48.2°
32	0.072	+49.0°
48	0.076	+51.6°
52	0.076	+51.6°
56	0.076	+51.6°

ear (arterial blood). While at the first examination of these patients (Table I, Columns 6 and 7) no difference was found between the amount of glucose in the venous and arterial blood

decided difference was observed in all cases after the administration of insulin, as will be seen from Table VI.

It follows from these investigations *that after the administration of insulin the patients are able to remove glucose from the blood during its passage through the capillary system at a time when new-glucose can be detected in the blood.*

As previously stated, these five patients were all examined at the same time (2 hours after their dose of insulin). It would be very interesting to study further the effect of a single insulin dose on the glucose of the blood partly to discover at what time after the insulin is given new-glucose appears in the blood, when the amount is greatest, and when the effect has completely disappeared, and partly by simultaneous investigation of the difference between the glucose content of the arterial and venous

TABLE VI.

Case No.	Glucose in cutaneous (arterial) blood.	Glucose in venous blood.	Difference between the glucose concentration in arterial and venous blood.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.188	0.178	0.010
2	0.459	0.430	0.029
3	0.241	0.203	0.038
4	0.087	0.074	0.013
5	0.298	0.294	0.004

blood, to find out whether there is a constant quantitative relation between the concentration of new-glucose in the blood and the amount of glucose which is removed from the blood stream at the same time, during its passage through the capillary system. These investigations, however, cannot be carried out by means of the technique hitherto employed, which requires too great a quantity of blood for series determinations. As previously mentioned, we are elaborating a technique which requires smaller amounts of blood, and we, therefore, hope to be able to investigate this problem later.

3. Patients with Benign Glycosuria.—We investigated two patients with glycosuria, which in accordance with the examination of the patients' fasting blood sugar and the course of the blood sugar curve after taking glucose, was considered to be

benign glycosuria. These two patients were investigated with the same technique as was employed in the earlier investigations, and the results are entered in Table VII. Both investigations were double determinations. Thus we find, on examining the dialysate immediately after dialysis, a diminished specific rotatory power for the glucose. It is considerably lower than the rotatory power of ordinary α,β -glucose and is of the same magnitude as the value found in normal persons. The rotatory power was followed as usual until it became constant and the values found at this time (after 48 hours) for the specific rotatory power approximated, as before, to that of α,β -glucose.

In these two patients with benign glycosuria new-glucose has thus been demonstrated in the blood. The ordinary examination of the glucose content of the arterial and venous blood (Columns 6 and 7, Table VII) show that in these patients at the time when the investigation was carried out, a quantity of glucose is removed during its passage through the capillary system.

DISCUSSION.

We will now, in conclusion, compare the results obtained from our various published works up to the present.

In our first papers, we showed that *in vitro* a new form of glucose was formed by adding insulin and fresh muscle tissue to α,β -glucose, for which we have proposed the name, new-glucose, until further knowledge of its chemical constitution is obtained. This form of glucose could always be detected in the blood of normal persons. Judging from our knowledge of its production *in vitro* we assume that it is also formed in the organism from the α,β -glucose absorbed from the intestinal canal with the aid of the pancreas hormone, insulin, and an unknown body or principle present in muscle.

We further assume that this form of glucose is the first step in the transformation of α,β -glucose in the organism.

In the present paper we have shown that in nine out of ten patients with diabetes mellitus new-glucose cannot be demonstrated in the blood. On the other hand, it was found in two diabetic patients who were much improved clinically after treatment. Five of these patients, in whose blood new-glucose was not found at the first examination, were investigated again after

the administration of insulin. It was then possible to detect new-glucose in the blood in every case. In two patients with benign glycosuria new-glucose was found in the blood. It was also found that *new-glucose* can be detected in all those cases where removal of glucose from the blood stream during its passage through the capillary system took place at the time the investigation was made. This was the case in (1) all the normal persons examined, (2) two patients with diabetes who were examined at the conclusion of the treatment, (3) five diabetics who were examined 2 hours after giving insulin, and (4) two patients with benign glycosuria. It was found, however, that in the nine patients with diabetes mellitus in whom new-glucose could not be demonstrated in the blood, no removal of the glucose from the blood during its passage through the capillary system took place, because simultaneous samples of arterial and venous blood showed no difference in glucose concentration.

We have thus shown that in all the normal and pathological cases investigated there is a close connection between the presence of new-glucose in the blood and the process by which it disappears from the blood during its passage through the capillaries. We regard it, therefore, as certain that new-glucose is an important link in the process of the breaking down of glucose in the organism and its reduction is an essential requirement for normal carbohydrate metabolism.

We will now briefly give our present view which we have formed from our experiments concerning the carbohydrate metabolism of the organism. In normal individuals the α, β -glucose absorbed from the intestine is changed into new-glucose by the pancreas hormone, insulin, and a substance present in the muscles. This transformation process is analogous to what took place in our experiments *in vitro*, but how this process comes about and where it takes place in the organism we still do not know. We assume that the new-glucose thus formed can then be broken down further in the organism. Our results so far do not permit us to draw any conclusion about the fate of the glucose arising from the decomposition of the proteins in the organism. We believe that that part of the glucose which disappears from the blood during its passage through the capillaries is new-glucose, but whether this substance is then deposited, for example, in the form of glycogen, or whether it is immediately oxidized in the tis-

sues, or whether perhaps both processes take place, cannot yet be decided.

In those *diabetics*, however, whose pancreas is so severely attacked that insulin is not secreted or only to a very small extent, the α,β -glucose absorbed from the intestine cannot be transformed. It will be found as such in the organism and when the amount in the blood exceeds a certain degree, it is excreted by the kidneys. If, on the other hand, *insulin* is administered to these patients the α,β -glucose can be transformed. Since in previous clinical investigations into the action of insulin in diabetes mellitus no case has ever been described where it is absolutely certain that insulin has failed to exhibit a therapeutic effect, we must assume that the substance in the muscles, which in our experiments *in vitro* proved to be just as important as insulin for the production of new-glucose, is either always present in diabetics, or at any rate present in the majority of diabetics.

In those diabetics in whom new-glucose was detected in the blood it was always shown that a removal of glucose from the blood took place during its passage through the capillary system. We believe, therefore, that insulin exerts this therapeutic effect in diabetics by changing α,β -glucose into new-glucose which is then transformed and broken down further in the organism. There is nothing in our previous results indicating that insulin acts at any other point in the transformation of glucose in the organism than this.

Without further discussing the old controversy whether the hyperglycemia in diabetes is due to a diminished combustion of carbohydrate (Minkowski) or an increased yield of carbohydrate from the liver (Von Noorden), or the converse problem which has arisen since the discovery of insulin, namely whether insulin acts by increasing the combustion of carbohydrate or diminishing the yield of carbohydrate from the liver, we can already point out that the results hitherto obtained by us definitely indicate that the hyperglycemia in diabetes arises as a consequence of the glucose absorbed from the intestine and of that formed by the decomposition of protein bodies not being broken down in the organism, and that the action of insulin consists in its causing an increased combustion of carbohydrate by transforming α,β -glucose into new-glucose which can then be further broken down in the organism.

TABLE VII.
Specific Rotatory Power of the Glucose in the Dialysate from the Blood of Two Patients with Benign Glycosuria.

Case No.	Date.	Sex.	Patient.	Age.	Glucose in ear blood.	Glucose in venous blood.	Glucose in dialysate immediately.		Specific rotatory power immediately.	Glucose in dialysate after 48 hrs.		Specific rotatory power immediately.	Remarks.
							Reduction.	Rotation.		Reduction.	Rotation.		
							per cent	(9)		per cent	(11)	(12)	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	per cent	(8)	(10)	per cent	(13)	(14)	
1	Oct. 2	♀	J. L.	18	0.128	0.116	0.056	0.031	+29.1°	0.056	0.055	+51.6°	$\frac{3}{4}$ hr. after 100 gm. glucose.
							0.057	0.032	+29.5°	0.057	0.056	+51.6°	
2	“ 3	♂	D. D.	42	0.231	0.207	0.122	0.079	+34.0°	0.122	0.118	+50.5°	$\frac{3}{4}$ hr. after 100 gm. glucose.
							0.116	0.074	+33.4°	0.115	0.111	+50.5°	

SUMMARY.

1. Out of eleven examinations on ten patients with severe or moderately severe diabetes mellitus, *new-glucose* could not be detected in the blood in nine. This substance, however, was found in two patients at the conclusion of the treatment.

2. In five of the patients in whose blood new-glucose could not be detected, it was present in the blood 2 hours after the administration of insulin.

3. In two patients with benign glycosuria, new-glucose was found in the blood.

4. In all the patients in whom new-glucose was detected in the blood a removal of glucose from the blood stream could at the same time be demonstrated during its passage through the capillaries, while this was not the case in diabetics where new-glucose could not be detected in the blood.

We therefore assume,

5. That new-glucose is an essential link in normal carbohydrate metabolism and probably the first, and

6. That insulin exerts its action in the organism in conjunction with a substance always or usually present in the muscles, by transforming α,β -glucose into new-glucose which can then be further broken down in the organism.

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STUDIES IN CARBOHYDRATE METABOLISM.

V. INVESTIGATIONS INTO THE FORM OF GLUCOSE IN DIFFERENT BODY FLUIDS.

By CHRISTEN LUNDSGAARD AND SVEND AAGE HOLBØLL.

(From Medical Clinic A, University of Copenhagen, Copenhagen, Denmark.)

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INTRODUCTION.

In our preceding investigations into the form of glucose in the blood of normal persons and diabetics, we employed dialysis through collodion membranes in order to provide ourselves with the glucose in a clear solution suitable for polarization determinations. With our technique hitherto employed this dialysis had to extend over a period of $1\frac{1}{2}$ hours in order to obtain a sufficiently large amount of glucose in the dialysate. As we have shown that the substance called by us *new-glucose*, which is characterized by a lower specific rotatory power than α,β -glucose, reverts to α,β -glucose in the dialysate in the course of 48 hours, it is, as previously stated, probable that a part of the new-glucose has already reverted to α,β -glucose during the dialysis. The result of this was that the specific rotatory values measured in the dialysate immediately after dialysis was at an end, must have been higher than the actual rotatory values for the glucose in the blood when the sample was taken.

By a method which will be described elsewhere we have subsequently succeeded in shortening the time of dialysis to half an hour; but even in this interval a part of the new-glucose will necessarily revert to α,β -glucose so that the values found in the dialysate will not absolutely coincide with the specific rotatory power of the glucose in the blood itself.

With the object of entirely dispensing with dialysis we have instituted experiments on the nature of the glucose in different body fluids, normal and pathological, investigating such as are naturally clear and therefore can be used immediately for polarization determinations.

EXPERIMENTAL.

Experimental Material.

Investigations were made of six spinal fluids, one edema fluid and one pleural fluid. They were all obtained from patients with normal carbohydrate metabolism. The examinations were made in connection with punctures carried out for some reason or other (diagnostic or therapeutic).

1½ hours previously the patients had taken 100 gm. of glucose by mouth, which had the effect of increasing the glucose concentration in the blood and therefore in the fluids examined.

Experimental Technique.

Immediately after the puncture was made, 2 pro mille sodium fluoride was added to the fluid to prevent possible glycolysis. A portion of the fluid was then put in the polarization tube and the rotation value read. This could be done directly with all the spinal and edema fluids. The pleural fluid, however, was not clear enough to be directly used for polarization determinations. It was, therefore, cleared with animal charcoal, and after filtration it was absolutely transparent and suitable for use. This procedure took about 10 minutes. The reduction value was determined as the mean of four analyses by Hagedorn and Norman Jensen's method on that portion of the fluid which was not employed for the rotation determination. The spinal fluid was also examined in the usual way for cells, albumin, and globulin. The rotation value was read at suitable intervals until constant values were obtained; the reduction value was then determined again.

Experimental Results.

All the results are entered in Table I. In the second and third columns the values of the reduction and rotation powers determined immediately after the fluid was taken, are given. As will be seen, there is in all cases a lower or even considerably lower value after the rotation determination. This is most clearly marked in the values calculated for the specific rotatory power, which in the case of the spinal fluid varies between +13.2° and +23.6°; for the edema fluid, it is +10.4°; and for the

pleural fluid, $+32.6^{\circ}$. The reduction values for the glucose in the spinal fluid are, as is known, lower than the values in the blood, so that the results found are of the same order as those obtained in the dialysate from the blood, although a considerable dilution has taken place in the latter case.

As previously mentioned, however, the difference observed between the rotation and reduction values is not in itself a proof that the glucose is present in a form which has a lower specific rotatory power, because the same might occur with the presence of other substances in the fluid which possessed either reducing prop-

TABLE I.

Investigations into the Reduction Power and Rotatory Power of Glucose in Different Body Fluids.

Kind of fluid.	Examined immediately after the sample was taken.			Examined after 48 hrs.		
	Glucose.		Specific rotatory value.	Glucose.		Specific rotatory value.
	Reduction.	Rotation.		Reduction.	Rotation.	
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
Spinal Fluid I.....	0.071	0.019	$+14.1^{\circ}$	0.071	0.068	$+50.1^{\circ}$
“ “ II.....	0.075	0.021	$+14.8^{\circ}$	0.074	0.071	$+50.3^{\circ}$
“ “ III.....	0.083	0.037	$+23.6^{\circ}$	0.083	0.080	$+50.2^{\circ}$
“ “ IV.....	0.069	0.030	$+22.4^{\circ}$	0.069	0.067	$+51.1^{\circ}$
“ “ V.....	0.068	0.021	$+18.1^{\circ}$	0.067	0.067	$+52.5^{\circ}$
“ “ VI.....	0.074	0.018	$+13.2^{\circ}$	0.074	0.072	$+50.9^{\circ}$
Edema “.....	0.072	0.014	$+10.4^{\circ}$	0.072	0.070	$+51.4^{\circ}$
Pleural “.....	0.058	0.036	$+32.6^{\circ}$	0.057	0.055	$+50.7^{\circ}$

erties or had the power to alter the plane of polarized light. One would think especially of the latter contingency when dealing with fluids like those investigated which have, of course, a small protein content which is generally stated to be slightly levo-rotatory in solution. The spinal fluids, however, contained only trifling amounts of protein. The edema fluid did not give Heller's reaction, but the pleural fluid showed a slight trace with this test.

A definite proof that we are dealing with the presence of a form of glucose with a lower specific rotatory power is furnished, however, by following the rotation value. It is found in all cases that the rotation value rises slowly and only reaches constant

values after the lapse of 48 hours. At this point the reduction power was again determined and the values for it and for the rotation value read simultaneously, are entered in the fifth and sixth columns in Table I. It was found that the reduction value remained unaltered, which shows that no loss of glucose had occurred. The rotation values, however, were considerably higher than the values read immediately and approximated closely to the values reached by the reduction power although they were always a little lower. This rise in the rotation value is best seen in the calculated values of the specific rotatory power which now lie close to and only a little lower than $+52.5^\circ$.

TABLE II.

Mutarotation of the Glucose in Spinal Fluid III.

Time after withdrawal.	Rotation value of glucose read.	Specific rotatory power.
<i>hrs.</i>	<i>per cent</i>	
0	0.037	$+23.6^\circ$
4	0.045	$+27.0^\circ$
8	0.048	$+30.3^\circ$
24	0.067	$+42.5^\circ$
28	0.071	$+44.8^\circ$
32	0.073	$+46.3^\circ$
48	0.080	$+50.2^\circ$
52	0.080	$+50.2^\circ$
56	0.080	$+50.2^\circ$

The fact that the theoretical value, $+52.5^\circ$, is not attained may either be explained in the same way as it was in the case of the dialysate from the blood, by the presence in the fluid of small quantities of other reducing substances than glucose (*e.g.* uric acid and creatinine), or by the occurrence of a small quantity of levorotatory substances (protein substances) which will decrease the rotation value. The difference between the reduction and rotation values is probably due to both factors together, but, as appears from the table, it is so small that we can take the figures given in the fourth column to be the true values for the specific rotatory power of glucose in the fluids investigated at the moment the samples were withdrawn.

In Table II the mutarotation of Spinal Fluid III is recorded.

The others showed an identical course. In Fig. 1 the mutarotation of the glucose in the spinal fluid is represented graphically together with the curve of the glucose in the dialysate from blood and of the new-glucose prepared *in vitro*. In addition the course of the mutarotation of chemically prepared β -glucose is recorded.

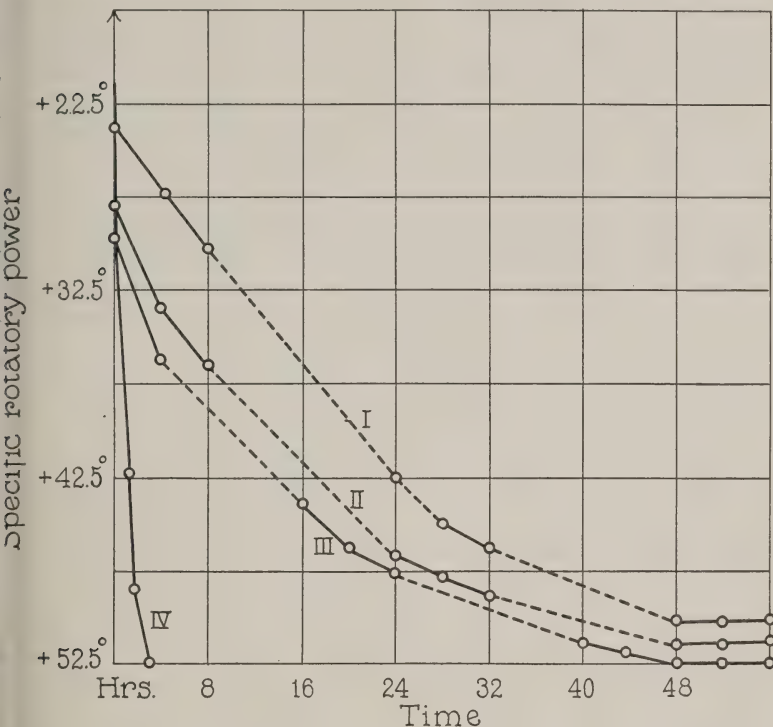


FIG. 1. The course of the mutarotation for the glucose in: Curve I, spinal fluid; Curve II, dialysate from blood; Curve III, new-glucose prepared *in vitro*; and Curve IV, β -glucose. Neutral reaction. Temperature 18°C .

All the determinations were made under similar external conditions; namely, at a temperature of 18° and in neutral reaction. It clearly appears from the curves that the course of the mutarotation is identical for the glucose in the spinal fluid, the glucose in the dialysate from blood, and the new-glucose prepared *in vitro*, but diverges greatly from that of β -glucose.

These experiments, therefore, prove that "new-glucose" can also be demonstrated in spinal fluid, edema fluid, and pleural fluid from patients with normal carbohydrate metabolism.

DISCUSSION.

The values found for the specific rotatory power of the glucose immediately after the withdrawal of the fluids are on the whole lower than those found in the dialysate from the blood of normal persons, which means that the percentage content of new-glucose is greater as compared with α,β -glucose in the tissue fluids than in the dialysate from blood. While in our investigations on blood we only observed a specific rotatory power which was lower than β -glucose's initial value of $+19^\circ$ in a single experiment, we found it in four out of the six spinal fluids and in the edema fluid. These results, therefore, afford direct experimental proof that the values obtained for the specific rotatory power cannot be explained by a simple shifting of the α - and β -glucose components in ordinary glucose, because values lower than $+19^\circ$ could never be reached in this way. These results, therefore, are a confirmation of our previous investigations on this subject.

How shall we explain the specially low values of the specific rotatory power, or in other words, to what is the high percentage concentration of new-glucose in the spinal and edema fluids due?

We may suppose that by using the natural dialyzing membrane of the body whereby we have avoided an artificial dialysis lasting $1\frac{1}{2}$ hours, none of the new-glucose has reverted to the α, β form. It is certain, as will be seen from Fig. 1, that some transformation takes place in the first $1\frac{1}{2}$ hours after withdrawal. There is, however, another possible way of explaining the results; namely, that new-glucose occurs in a higher percentage concentration in the tissue fluids than in the blood. This result, again, might arise in different ways. We might assume that the production of new-glucose occurs in blood, but that it is taken up and bound by the tissue fluids to a marked degree, or we can suppose that the transformation only takes place in the tissues, and the tissue fluids therefore contain new-glucose in greater amounts than the blood. In favor of this last explanation there is the previously discovered fact that new-glucose is only formed from α,β -glucose by the simultaneous action of insulin and a substance demonstrated by us which is only present in an active form in fresh muscle tissue.

SUMMARY.

1. Determinations of the specific rotatory power of the glucose in spinal, edema, and pleural fluids from patients with normal carbohydrate metabolism have been made. Such determinations can be made immediately (without the preliminary dialysis required for blood) because the fluids themselves are suitable for polarization determinations.
2. New-glucose was found in all the cases investigated and, as regards the spinal and edema fluids, in greater concentration than occurs in the dialysate from blood.
3. This greater concentration of new-glucose may be due to the facts that dialysis is avoided and the determinations are made, therefore, before the mutarotation had begun, or to the fact that the concentration of new-glucose is greater in the tissue fluids than in the blood.

THE ELECTRODIALYSIS OF AGAR.

A METHOD FOR THE PREPARATION OF THE FREE AGAR-ACID.*

By WALTER F. HOFFMAN AND ROSS AIKEN GORTNER.

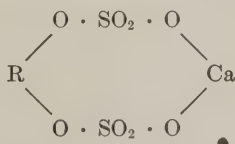
(From the Division of Agricultural Biochemistry, University of Minnesota,
St. Paul.)

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The chemical constitution of agar is not definitely known. It is usually referred to as γ -galactan. The organic material consists chiefly of a hemicellulose which, on hydrolysis, yields a large percentage of *d*-galactose. Besides this sugar a small amount of other hexoses and pentoses are also formed. The ash content of even highly purified agar samples is very high. Fellers (1) has shown that the ash is mostly calcium sulfate together with traces of other salts.

It is impossible to remove all of the ash from agar by dialysis. Samec and Ssajevič (2) found that after dialyzing agar for 3 months, it still contained a considerable amount of inorganic matter. Pascheles (3) and MacDougall and Spoehr (4) claim to have used practically salt-free or ashless agar, but do not give analyses to support their statements.

Haas (5), while working on carrageen (*Chondrus crispus*), has shown that the ash content cannot be reduced, by dialysis, below 14.6 per cent. This ash was found to be principally calcium sulfate. In solutions of carrageen, calcium ions were found to be present, but no sulfate ions. When ashed, only about half of the sulfur as sulfate was recovered, the rest being lost during ignition. Haas concludes that the calcium and the sulfate are integral parts of the carrageen molecule and are present as a sulfuric ester.



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Fairbrother and Mastin (6) also present evidence that agar consists principally of the calcium salt of an acid sulfuric ester $(R \cdot O \cdot SO_2 \cdot O)_2Ca$. They found that the effect of acid on agar is to produce a reversible equilibrium of the type $(R \cdot O \cdot SO_2 \cdot O)_2Ca + 2 HCl \rightleftharpoons 2 R \cdot O \cdot SO_2 \cdot OH + CaCl_2$ the calcium salt and its free acid sulfuric ester being ionized to some extent. Besides such physical properties as swelling, etc., they give as further evidence in support of their theory of the composition of agar gel, its behavior as an electro-osmotic diaphragm. No reversal of the direction of flow takes place when the diaphragm is acidified. They suggest that in the neutral state, the micellæ consist of undissociated salt and sulfuric ester ion and that when acid is added, the calcium is partly replaced by hydrogen and the product remains still electronegative. In the presence of alkali, the calcium is partly replaced by the alkali metal. "During all of these changes the colloid complex remains negatively charged with respect to water, and therefore the direction of the electroendosmosis will be toward the cathode."

Attempts were made by Fairbrother and Mastin to prepare the free acid $R \cdot O \cdot SO_2 \cdot OH$ by soaking agar in dilute hydrochloric acid and water alternately. By this procedure a gel was obtained which was ash-free except for silica. When this gel was dissolved in water by the aid of heat, it could not be made to gel again even after long standing. Samec and Isajević (7) have found that when agar is purified by dialysis or even by electrodialysis there is a large amount of electrolytes (principally sulfuric acid) which are not removed from agar. The sulfuric acid could be removed by dialysis if the solution had been previously heated under pressure. This loss of sulfur is attributed to a hydrolysis and produces a marked change in the physical properties of the agar.

The calcium portion of the agar molecule appears to be largely ionic calcium, $Ca (R \cdot O \cdot SO_2 \cdot O)_2 \rightarrow Ca^{++} + 2 (R \cdot O \cdot SO_2 \cdot O)^-$. It has been shown that the calcium in agar can be replaced by other metals. Fairbrother and Mastin (6) were able to prepare a potassium agar by heating agar with a slight excess of potassium oxalate. The quantity of potassium in the agar was approximately equivalent to the calcium which was originally present. The potassium agar sol filtered readily and set to a rigid gel.

That the sulfate is tightly bound or combined in the molecule as an ester is demonstrated by the fact that none of the sulfur is in the form of SO_4 ions in the original agar, but when the agar has been hydrolyzed by boiling with 10 per cent hydrochloric acid practically all of the sulfur is present as sulfuric acid. The work of Haas and Russell-Wells (8) on the ethereal sulfates of marine algæ further support this. From their data it appears that a large number of the marine algæ have calcium sulfate occurring in some sort of chemical combination with an organic complex. This combination is probably in the form of an ethereal sulfate. Regarding this they state: "The ethereal sulfates above referred to have their metallic constituent freely ionized but the sulfate is masked by being in combination with a complex colloidal aggregate; they accordingly belong to the group of colloidal electrolytes and as such have a measurable conductivity and exhibit osmotic phenomena."

Harvey (9) has recently published a note on the electrodialysis of agar. We were not aware of this publication until the present manuscript had been completed. He gives no analyses for sulfur or for the individual constituents of the ash, and the hydrogen ion concentration of his final product (pH 4.9) indicates that he did not carry the electrodialysis to completion. The greater part of his paper is devoted to a study of the viscosity of the electrodialyzed agar.

From the evidence presented in the work briefly outlined above, it seemed desirable to study the effect of prolonged electrodialysis on agar, measuring quantitatively the effects of hydrolysis if any occurs, and to study the physical and chemical properties of the resulting product.

EXPERIMENTAL.

For these experiments, Bacto-Agar was used. It contained 1.39 per cent of moisture. The percentages of certain ash constituents, calculated to the dry basis, are shown in Table II.

A three compartment box as suggested by Foster and Schmidt (10) was employed as a dialyzing vessel. The compartments had a capacity of 1250 cc. each. They were separated by heavy sheets of collodion supported by a double layer of light canvas. This provided a very suitable, durable, and semipermeable membrane. Even after many days use they were in perfect condition. Large carbon plates (12 × 15 cm.) were used for electrodes. The current was 220 volts (D.C.) and the amperage was as high as it was possible to obtain and was limited only by the conductance of the liquid between the electrodes.

50 gm. of coarsely ground air-dry agar were suspended in 1250 cc. of distilled water and placed in the middle compartment. The dialysis was carried out as shown in Table I, which contains the record of the length of dialysis, amperage, and temperature. The temperature did not exceed 50°C. at any time and was never allowed to remain at this point for more than a few minutes. The 1250 cc. of water in the end compartments were removed at the end of each hour period and replaced by an equal amount of distilled water. The water removed from the middle compartment by electroendosmosis was replaced by distilled water.

The water removed from the end compartments was tested for sulfates and total sugars, but none of the samples gave a positive

test for sugars or sulfur. The calcium migrated to the anode compartment, but due to the low concentration at any one time quantitative estimations were not attempted.

It was not possible, during the first few hours to obtain a current greater than about 0.2 ampere, but after some hours dialysis the current increased to more than 1 ampere. The amount of current being directly proportional to the conductance of the contents of the middle compartment is indicative of the relative

TABLE I.
Data on the Electrodialysis of Agar.

Temperature of center compartment.	Length of dialysis.	Amperes.	Ampere-hrs.
°C.	hrs.		
22-25	1	0.1-0.2	0.15
21-24	1	0.2-0.2	0.20
23-28	1	0.2-0.2	0.20
22-35	1	0.2-0.5	0.35
21-32	1	0.4-0.5	0.45
23-36	1	0.6-0.6	0.60
23-31	1	0.4-0.4	0.40
25-34	1	0.3-0.4	0.35
21-34	1	0.5-0.5	0.50
24-36	1	0.4-0.6	0.50
24-36	1	0.6-0.8	0.70
28-40	1	0.6-0.8	0.70
20-40	1	1.0-1.2	1.10
30-43	1	0.8-1.0	0.90
30-50	1	1.0-1.3	1.15
20-50	1	1.0-1.2	1.10
26-50	1	1.2-1.4	1.30
28-40	1	1.0-1.0	1.00
Total.....	18		11.65

resistivity of the calcium salt of agar and of the free acid. This is additional proof that agar is a calcium salt and that it ionizes or dissociates into a calcium ion and an organic ion resulting in a rather poor conductor of an electric current, but as the calcium ions are removed from the solution and the free acid is formed the conductors are hydrogen ions, which have a high transport number as compared to calcium ions, and the organic ions. This change from the calcium salt to the free acid increases the conduc

tivity approximately six times which is in the same order as the transport numbers. Tables of physical constants show the mobilities of the hydrogen and calcium ions at 18°C. to be 315 and 51 (for $\frac{1}{2}$ Ca) respectively.

After electro dialyzing this agar suspension for 18 hours, the contents of the middle compartment were removed. No gelation had occurred and the original granules still remained but were greatly swollen and translucent. The liquid in which they were suspended contained 0.7 per cent of dry matter apparently in solution, and showed no appreciable viscosity.

When this solution, or the agar particles filtered from this solution, was dried at 60°C. the dried residue apparently charred and became coal-black. This was probably due to the high acidity of the free "agar-acid." This "char" was readily soluble in hot water, forming a deep caramel-colored solution, but such solutions even when they exceed a 4 per cent agar had but slight viscosity and showed no tendency to gelatinate on standing.

The agar particles filtered from the electro dialyzed solution could be dried at 30° without causing discoloration, the resulting product closely resembling the original agar. A 5 per cent solution of this product was but slightly viscous and showed no tendency to gelatinate on cooling. On the contrary, simply heating his product with water caused very appreciable hydrolysis as indicated by the formation of reducing sugars.

The aqueous solution (0.7 per cent of dry matter) from the center compartment is a most efficient "protective colloid." Using this solution as a protective colloid, it was possible to prepare metallic sols of silver chloride, silver hydroxide, barium sulfate, etc., which were extremely stable and which showed no settling even after many days standing. The ease with which a metallic colloidal sol can be prepared in the presence of this dialyzed agar would make it a valuable adjunct to colloid research.

An exact aliquot of the suspension of agar in the liquid in the center compartment was diluted to a known volume and the following analyses were made on this solution.

After precipitating the agar, etc., from the equivalent of 2 gm. of the original agar, with lead acetate, and removing the lead with potassium oxalate, tests for reducing sugars were entirely negative, showing that electro dialysis had not hydrolyzed the carbohydrate portion of the agar.

Another sample containing the same amount of original agar was diluted to about 150 cc. and heated in a boiling water bath for $2\frac{1}{2}$ hours. The solution was clarified as described above and reducing sugars were determined according to the method described by Quisumbing and Thomas (11). 90 mg. of reducing sugar, calculated as glucose, were obtained from the 2 gm. of air-dry agar or 101.5 mg. of glucose on the dry basis.

A sample containing the same amount of agar was treated exactly as the sample described above except that enough hydrochloric acid was added to make a 1.5 per cent solution. 719 mg. of reducing sugars, calculated as glucose, were obtained from the 2 gm. of original agar or 811 mg. of glucose on the dry basis.

These analyses show that the electrodialysis did not hydrolyze the agar to reducing sugars. The acidity of the free acid was sufficient to cause appreciable autohydrolysis on heating. This confirms the qualitative tests of Samec and Isajević on their electrodialyzed agar and of Fairbrother and Mastin (6) on their ashless agar-acid.

The results of analyses of the dialyzed agar for certain constituents are given in Table II. From these data it is evident that during the dialysis the calcium is removed quantitatively from the agar leaving the free acid. The sulfur is combined with the carbohydrate radicle and is not removed by the dialysis. This is additional evidence of a calcium salt of a sulfuric acid ester being the formula of the agar molecule. Very little of the silica is removed by dialysis. This is to be expected if it is present in colloidal form. There is no evidence that it is chemically combined in the agar molecule.

The free acid obtained by electrodialyzing agar is a stronger acid than most organic acids. The dialyzed agar suspension before dilution showed an acidity equal to pH 2.0 and when diluted to a 1 per cent¹ solution had a hydrogen ion concentration of pH 2.475 which is equivalent to about 0.003 N sulfuric acid. The acidity is so great that no mould or bacterial growths have developed on solutions which have stood uncovered in the laboratory for several months.

¹ The term "1 per cent solution," as used in this paper, refers to a concentration equivalent to 1 per cent of the original air-dry agar which was used for the electrodialysis. The actual concentration of the dry free agar-acid in this solution was approximately 0.78 per cent.

Table III presents the results of an electrometric titration of this dialyzed agar. The free acid ionizes as a strong acid, since almost all of the acid is neutralized below pH 4. The data

TABLE II.

Analysis of the Dialyzed Agar and Normal Agar for Some Inorganic Constituents.

	Normal agar.*	Dialyzed agar.*
	<i>per cent</i>	<i>per cent</i>
Ash.....	4.66	0.70
Sulfur (S).....	1.01	1.01
Calcium oxide (CaO).....	0.99	0.04
Silica (SiO ₂).....	0.53	0.57

* Calculated on the basis of the original dried agar.

TABLE III.

Electrometric Titration of 200 Cc. of 1 Per Cent† Electrolyzed Agar with Sodium Hydroxide.*

NaOH	N	E.M.F.	pH	C _H
<i>cc. 0.1N</i>		<i>mv.</i>		
		430.0	2.475	0.00327
1.0	0.0005	432.0	2.519	0.00303
2.0	0.0010	434.5	2.562	0.00274
3.0	0.0015	437.5	2.611	0.00245
4.0	0.0020	441.0	2.671	0.00213
5.0	0.0025	445.5	2.747	0.00179
6.0	0.0030	459.0	2.975	0.00106
7.0	0.0035	471.5	3.187	0.000651
8.0	0.0040	484.5	3.407	0.000393
9.0	0.0045	525.0	4.091	0.811×10^{-4}
10.0	0.0050	607.0	5.478	0.333×10^{-5}
11.0	0.0055	847.0	9.535	0.292×10^{-9}

* The method as previously described by the authors was used. Cf. second national colloid symposium monograph, New York, 1924, Chapter 20, p. 209-368.

† Calculated on basis of 1 per cent of the original air-dry agar.

indicate that the free acid is not completely ionized even when diluted to a 1 per cent solution. The concentration of the acid in a 1 per cent solution as calculated from the sulfur content (air-dry basis) should be 0.0056 N (calculating on the assumption that

$R \cdot O \cdot SO_2 \cdot OH$ is the formula of the free acid.) This agrees very well with the experimental value inasmuch as it requires an equal amount of 0.0050 N sodium hydroxide solution to neutralize a 1 per cent solution of the agar-acid. These values show that the actual acidity (hydrogen ion concentration) is only about 56 per cent of the titratable acidity. Apparently the acid hydrogens of the agar-acid are less active than are those in the free sulfuric acid but more active than are those in the usual "organic acids."

To ascertain whether or not calcium is specific for forming an agar salt that will readily set to a rigid gel, different salts of the free acid were prepared. The following inorganic bases were used: sodium hydroxide, potassium hydroxide, calcium hydroxide, magnesium oxide, barium hydroxide, strontium hydroxide, and lithium carbonate. Enough of the base was added to just neutralize the free acid (as calculated from the titration curve of the free agar-acid) and the mixture heated in a water bath for a few minutes until the agar was completely melted. After a few hours the agars all set to rigid gels. There appeared to be no pronounced difference in the consistency of the gels formed by the salts of the mono- and bivalent metals.

A few organic bases were tried, *e.g.* aniline, urea, and the alkaloids, strychnine and cinchonidine. Urea formed a very soft gel but aniline and the alkaloids formed rigid gels, the alkaloid gels being apparently firmer than the gels of the metal salts. Possible pharmacological uses for the agar-alkaloid salts are being investigated.

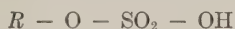
SUMMARY.

1. Commercial agar was electrodialyzed during a total period of 18 hours, using a total electric current of approximately 11.65 ampere-hours at a potential of 220 volts.
2. Such electrodialysis removed the calcium practically quantitatively and formed the free agar-acid.
3. No sulfur was removed by electrodialysis.
4. A 1 per cent solution of the free agar-acid had a hydrogen ion concentration of pH 2.475. The acid is apparently about 56 per cent ionized and is neutralized by sodium hydroxide below a pH of 4.0.
5. The free agar-acid is apparently an acid sulfuric acid ester. All of the sulfur of agar is in the form of sulfuric acid.

6. Sols of the free agar-acid containing as much as 5 per cent of the agar-acid do not gelatinize upon cooling. Appreciable autohydrolysis takes place when sols of the agar-acid are heated.

7. When sols of the free agar-acid are neutralized by the addition of a base, rigid gels are obtained. Organic bases such as aniline and the alkaloids form salts with the agar-acid and set to rigid gels.

8. A rough calculation derived from the titration data, the percentage of alkaloid in the alkaloid compounds, and the sulfur content indicate that the minimum molecular weight of the free agar-acid is in the neighborhood of 3000 and probably corresponds to the formula



where R is a large polysaccharide residue.

9. The gelation of agar is the gelation of a salt and not the gelation of a complex polysaccharide.

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STUDIES OF ACIDOSIS.

XXI. THE COLORIMETRIC DETERMINATION OF THE pH OF URINE.

By A. BAIRD HASTINGS, JULIUS SENDROY, JR., AND WILLIAM ROBSON.*

(From the Hospital of The Rockefeller Institute for Medical Research.)

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Colorimetric methods for the determination of urine pH have been described by Henderson and Palmer (1), Michaelis (2), and others, which are sufficiently accurate for most clinical purposes. Quagliariello and D'Agostino (3) proposed a colorimetric method, which checked their electrometric determinations with an error ranging from -0.16 to $+0.15$ pH. They employed phosphate buffers as standards, with neutral red and para-nitrophenol indicators. The temperature was not controlled, nor was the dye concentration in the standard and unknown solutions uniform throughout the range of any one indicator. Apparently no attempt was made to prevent loss of CO_2 from urine samples, or to take into consideration the partial pressure of this gas in the electrometric measurements. Marshall (4) has shown that the loss of CO_2 from urine specimens may cause quite an appreciable error in pH determinations, amounting to several tenths of a pH in some alkaline samples.

In the present paper we have attempted to increase the accuracy of the colorimetric method, and have controlled it by comparison with electrometric measurements. Due to reasons which will be pointed out, the accuracy of the method, even with the precautions employed, is only 0.1 pH as compared with an accuracy of ± 0.02 pH in the case of blood determinations. The bicolor standards, used by Hastings and Sendroy (5) for blood plasma pH measurements, have been adapted to urine.

* Fellow in Research Medicine of The Rockefeller Foundation.

Method.

The urine is collected and kept under oil. 2 cc. are pipetted without exposure to air into a test-tube containing 1 cc. of indicator solution and 7 cc. of redistilled water under oil. As a control tube, another 2 cc. portion is run into a similar test-tube with 8 cc. of water. After gently stirring with a footed rod, the unknown solution is brought to 38° in a water bath and matched with pairs of standard indicator solutions as previously described (5). To allow for the error introduced by dilution of the urine, 0.10 pH is subtracted from the reading at 38°. This corrected pH has been found to be within 0.05 of the electrometric pH at the same temperature.

The apparatus required is an adequate number of clear glass test-tubes (22 × 175 mm.) of uniform inside diameter, a calibrated micro burette graduated to 0.02 cc., a 50 cc. burette, and a 3-row comparator block.

Preparation of the Color Standards.—The color standards used for comparison with the unknown are prepared as outlined in Tables I to IV.

Each pH standard consists of two tubes, one of which contains a known concentration of the indicator in its alkaline form, the other in its acid form. The concentrations are so chosen that the sum of the concentrations of indicator in the two tubes of each pair is constant throughout the series.

This method has been used by Bjerrum, Michaelis, Sørensen, Gillespie, Myers, and others. It is based on the assumption that the color of an indicator solution is due to a mixture of the acid and alkaline forms of the indicator. Each form has its peculiar color, and the proportions of the two forms are related to the reaction of the solution as indicated by the Henderson-Hasselbalch equation for solutions of buffer acids and their salts, *viz.*

$$\text{pH} = \text{pK}' + \log \frac{\text{BA}}{\text{HA}}$$

where BA is the alkaline salt, in this case the alkaline form of the indicator, and HA is the free acid, or the acid form of the indicator. Variations in the ratio of the alkaline form to the acid form correspond to variations in pH according to the above equation.

Values for the pK' of the indicators used at room temperature have been determined by Clark (6), Gillespie (7), Brode (8), and Holmes and Snyder (9), and have been verified by the authors. Additional values of pK' at 38° have also been determined by the authors.

The quantities of indicator solution to be added to each tube are accurately measured from a micro burette. To each tube

TABLE I.

Table for Preparation of Bicolor Standards, with 0.016 Per Cent Brom Cresol Green, 0.002 N HCl, and 0.001 N NaOH. Brom Cresol Green $pK' = 4.72$ at 38° and 20° .

$pH_{38^\circ \text{ and } 20^\circ}$	Alkali tube.		Acid tube.	
	cc. dye	cc. alkali	cc. dye	cc. acid
4.00	0.40	24.60	2.10	22.90
4.10	0.49	24.51	2.01	22.99
4.20	0.58	24.42	1.92	23.08
4.30	0.69	24.31	1.81	23.19
4.40	0.81	24.19	1.69	23.31
4.50	0.94	24.06	1.56	23.44
4.60	1.08	23.92	1.42	23.58
4.70	1.23	23.77	1.27	23.73
4.80	1.38	23.62	1.12	23.88
4.90	1.51	23.49	0.99	24.01
5.00	1.64	23.36	0.86	24.14
5.10	1.77	23.23	0.73	24.27
5.20	1.88	23.12	0.62	24.38
5.30	1.98	23.02	0.52	24.48
5.40	2.07	22.93	0.43	24.57
5.50	2.14	22.86	0.36	24.64
5.60	2.21	22.79	0.29	24.71
5.70	2.26	22.74	0.24	24.76
5.80	2.31	22.69	0.19	24.81

either dilute acid or alkali is then added to make a total volume of 25 cc. (Due to its greater stability, 0.001 N HCl has been substituted for the 0.0001 N HCl originally prescribed (5) for the acid tubes of the phenol red series.) The tubes are stoppered or sealed, and kept in a dark cupboard. When sealed, the solutions are stable for several months.

Preparation of Indicator Solutions.—The indicators, brom cresol green, brom cresol purple, and phenol red, covering a pH

TABLE II.

Table for Preparation of Bicolor Standards with 0.01 Per Cent Chlor Phenol Red, 0.001 N HCl, and 0.01 N NaOH. Chlor Phenol Red $pK' = 5.93$ at 38° , and 6.02 at 20° .

pH_{38°	Alkali tube.		Acid tube.		pH_{20°
	cc. dye	cc. alkali	cc. dye	cc. acid	
5.00	0.26	24.74	2.24	22.76	5.09
5.10	0.32	24.68	2.18	22.82	5.19
5.20	0.39	24.61	2.11	22.89	5.29
5.30	0.48	24.52	2.02	22.98	5.39
5.40	0.57	24.43	1.93	23.07	5.49
5.50	0.68	24.32	1.82	23.18	5.59
5.60	0.80	24.20	1.70	23.30	5.69
5.70	0.93	24.07	1.57	23.43	5.79
5.80	1.07	23.93	1.43	23.57	5.89
5.90	1.20	23.80	1.30	23.70	5.99
6.00	1.35	23.65	1.15	23.85	6.09
6.10	1.50	23.50	1.00	24.00	6.19
6.20	1.63	23.37	0.87	24.13	6.29
6.30	1.75	23.25	0.75	24.25	6.39

TABLE III.

Table for Preparation of Bicolor Standards with 0.008 Per Cent Brom Cresol Purple, 0.002 N HCl, and 0.01 N NaOH. Brom Cresol Purple $pK' = 6.09$ at 38° , and 6.19 at 20° .

pH_{38°	Alkali tube.		Acid tube.		pH_{20°
	cc. dye	cc. alkali	cc. dye	cc. acid	
5.60	0.61	24.39	1.89	23.11	5.70
5.70	0.72	24.28	1.78	23.22	5.80
5.80	0.85	24.15	1.65	23.35	5.90
5.90	0.99	24.01	1.51	23.49	6.00
6.00	1.12	23.88	1.38	23.62	6.10
6.10	1.26	23.74	1.24	23.76	6.20
6.20	1.40	23.60	1.10	23.90	6.30
6.30	1.55	23.45	0.95	24.05	6.40
6.40	1.68	23.32	0.82	24.18	6.50
6.50	1.80	23.20	0.70	24.30	6.60
6.60	1.91	23.09	0.59	24.41	6.70
6.70	2.01	22.99	0.49	24.51	6.80
6.80	2.09	22.91	0.41	24.59	6.90
6.90	2.16	22.84	0.34	24.66	7.00

range of 4.0 to 8.2, have been used. The dissociation curves of these dyes overlap each other within the useful range and they are probably the most suitable for urine work at this time. Brom cresol green, as recommended by Cohen (10), has displaced methyl red. Chlor phenol red may sometimes prove useful, when difficulty is experienced in reading the higher pH standards of brom cresol green. However, believing that the three other indicators fulfill most requirements, we recommend that chlor phenol red be used only when necessary. Al-

TABLE IV.

Table for Preparation of Bicolor Standards with 0.0075 Per Cent Phenol Red, 0.001 N HCl, and 0.01 N NaOH. Phenol Red $pK' = 7.65$ at 38° , and 7.78 at 20° .

pH _{38°}	Alkali tube.		Acid tube.		pH _{20°}
	cc. dye	cc. alkali	cc. dye	cc. acid	
6.70	0.25	24.75	2.25	22.75	6.83
6.80	0.31	24.69	2.19	22.81	6.93
6.90	0.38	24.62	2.12	22.88	7.03
7.00	0.46	24.54	2.04	22.96	7.13
7.10	0.55	24.45	1.95	23.05	7.23
7.20	0.65	24.35	1.85	23.15	7.33
7.30	0.77	24.23	1.73	23.27	7.43
7.40	0.90	24.10	1.60	23.40	7.53
7.50	1.04	23.96	1.46	23.54	7.63
7.60	1.18	23.82	1.32	23.68	7.73
7.70	1.32	23.68	1.18	23.82	7.83
7.80	1.46	23.54	1.04	23.96	7.93
7.90	1.60	23.40	0.90	24.10	8.03
8.00	1.73	23.27	0.77	24.23	8.13
8.10	1.85	23.15	0.65	24.35	8.23
8.20	1.95	23.05	0.55	24.45	8.33

though exhibiting dichromatism, brom cresol purple, in the absence of a better indicator covering the same range, is still indispensable.

Stock solutions of the indicators were made by dissolving 0.1 gm. of dye in 1 or 1.1 equivalents of NaOH, as recommended by Clark. For convenience these directions will be repeated. 0.1 gm. of each dye is ground in an agate mortar with the following quantities of 0.05 N NaOH.

Indicator.	Equivalents of NaOH to add.	0.05 N NaOH solutions to be added per 0.1 gm. of indicator.
		cc.
Phenol red.	1.0	5.7
Brom cresol purple.	1.1	4.1
Chlor phenol red.	1.1	5.2
Brom cresol green.	1.1	3.2

After the dye is completely dissolved in the alkali, the solution is transferred to a 100 cc. volumetric flask, and diluted to the mark. From these stock solutions the more dilute concentrations used in the determination of pH are prepared as needed. The latter are prepared by diluting the stock 0.1 per cent solutions as follows:

Indicator.	Final concentrations.	Stock solution diluted to 200 cc.
	per cent	cc.
Phenol red.	0.0075	15
Brom cresol purple.	0.008	16
Chlor phenol red.	0.01	20
Brom cresol green.	0.016	32

The above concentrations of dye are those found most convenient to read by Daylite lamp. However, slight variation in the concentration of the dye solution used does not affect the accuracy of the readings, provided the same dye solution is used in both standards and unknowns. Samples of indicators are sometimes found to contain an insoluble impurity. If this is but a small amount, it may be filtered off and disregarded.

EXPERIMENTAL.

Determinations of the pK' of Indicators at 38° and 20°.—As outlined in a previous communication (5), these values were determined by comparison of pairs of bicolor standards in dilute alkali and acid with standard tubes of M/15 phosphate or M/5 acetate mixtures containing dye in the same proportion. These buffer mixtures were prepared from Merck's salts, the acetate being recrystallized. The pH of phosphate and acetate solu-

tions was determined electrometrically at 20°, using as a standard of reference the ϵ of the saturated calomel electrode obtained with 0.1 N HCl, assuming a pH of 1.08 at 20°. A correction of -0.03 was made for the phosphate values at 38° while those obtained for the acetates at 20° were used also at 38°, there being no temperature coefficient according to Michaelis (11). From these determinations an average pK' for each indicator at 20° and at 38° was obtained, from which the theoretical dissociation curves were constructed (Fig. 1).

Tables I to IV give the amounts of dye solutions required for the bicolor standards as calculated from the pK' values determined in the above manner.

The constants obtained and others already in the literature are as follows:

Indicator.	pK'	Temperature.	Method.	Author.
Phenol red.		°C.		
	7.90	Room.	Spectrophotometric.	Brode.
	7.9	"	Colorimetric.	Clark.
	7.7	25-30	"	Gillespie.
	7.76	25-28	"	Wu (12)
	7.77	20	"	Earnett and Barnett (13)
	7.78±0.007	20	"	Hastings and Sendroy.
	7.65±0.009	38	"	" "
Brom cresol purple.	6.30	Room.	Spectrophotometric.	Brode.
	6.3	"	Colorimetric.	Clark.
	6.3	25-30	"	Gillespie.
	6.28	20	"	Barnett and Barnett.
	6.19±0.025	20	"	Authors.
	6.09±0.029	38	"	"
Chlor phenol red.	6.02±0.019	20	Colorimetric.	Authors.
	5.93±0.022	38	"	"
Brom cresol green.	4.68±0.01	27	Spectrophotometric.	Holmes and Snyder.
	4.72±0.012	20 and 38	Colorimetric.	Authors.

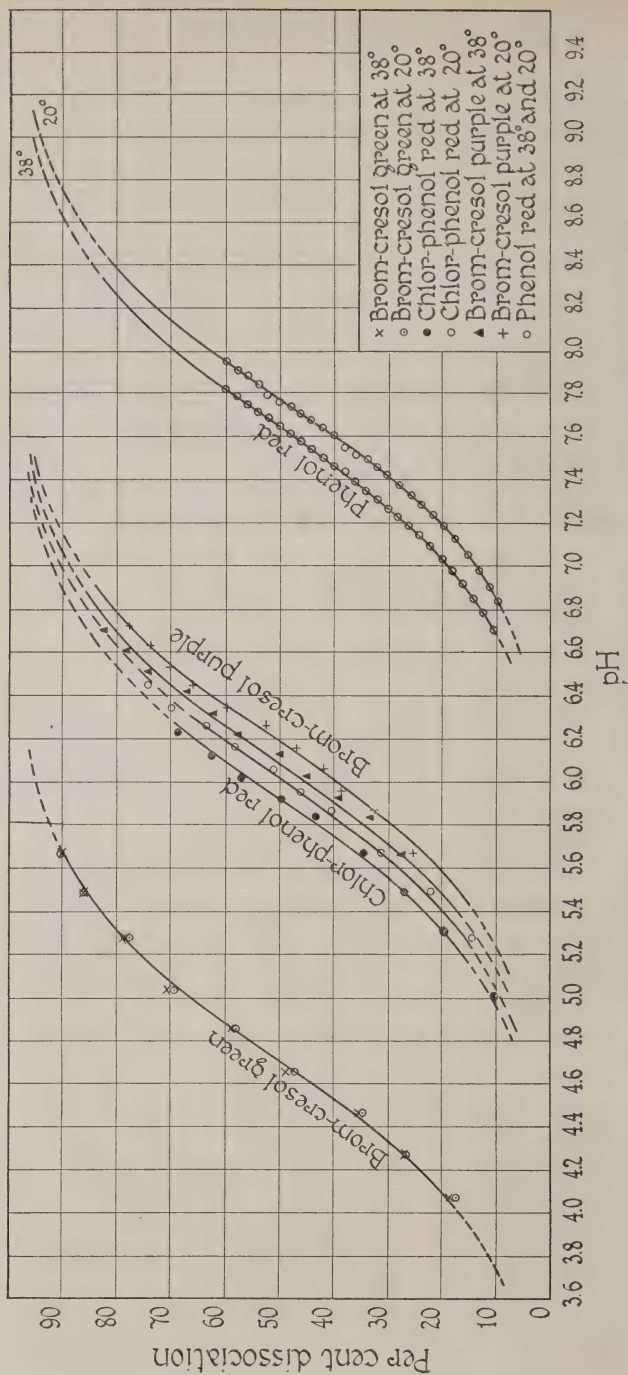


FIG. 1. Calculated and observed dissociation curves for indicators used in urine pH determinations.

While these are only apparent dissociation constants, they hold true for determinations carried out under the prescribed conditions. Due to the optical difficulties encountered in reading the colors of brom cresol purple and chlor phenol red, it has not been found possible to determine these constants with the same order of accuracy as that obtained with the other two. However, inasmuch as individual variations in different lots of dye in themselves may cause deviations comparable to those given above, and since the unknown salt concentration of the urine sample also introduces an error, the values given are thought to be sufficiently close to fall within the limits of error of the method. The dye concentration has been altered from twice to one-half that prescribed above, and in no case has there been any difference from the values given.

The tables given for the preparation of standards are based on the assumption that *the pure dye, of constant pK' throughout its useful range*, is being used. However, in the experience of other workers (8), individual lots of dye may have different apparent dissociation constants.

Of the several different samples of brom cresol green used in this laboratory, some have been found to be very poor. Samples of brom cresol green in the dry state varied in color from dark purple to an orange reddish tinge, and gave melting points such as 227°, 223°, and 215.5°. The latter, a sample from LaMotte Chemical Products Company, proved to be entirely satisfactory. The dibrom derivative, brom cresol purple, gave a melting point at 243°.

Effect of Dilution on pH of Urine.—Samples of normal urine were saturated twice at 38° with a gas mixture containing CO₂ at 40 mm. tension plus hydrogen to 1 atmosphere. After saturation, urine samples of various dilutions were analyzed electrometrically, with a CO₂ partial pressure corresponding to that of the diluted sample in each case; *e.g.*, if the urine was diluted 5-fold, hydrogen containing CO₂ at 8 mm. tension was used in the electrode vessel. Table V, Sample 1, gives some idea of the magnitude of change of pH taking place on dilution of urine. Samples 2 to 7 were analyzed as delivered under oil or aerated (2 and 3) with CO₂-free hydrogen in the electrode. In these samples the effect of dilution (indicated in Table V

as Δ pH) amounted to $+0.08 \pm 0.03$ pH. Due to the difference in nature and amount of salt in individual urine specimens

TABLE V.

Effect of Dilution on the pH of Urines Determined Electrometrically.

Sample No.	Temperature.	Dilution.	Electrometric pH undiluted.	Electrometric pH diluted.	Δ pH due to dilution.
1	38	0	5.46		
		2×		5.49	+0.03
		3×		5.52	+0.06
		6×		5.56	+0.10
		11×		5.59	+0.13
		16×		5.62	+0.16
2	20	5×	7.14	7.23	+0.09
3		5×	7.81	7.92	+0.11
4		5×	5.58	5.63	+0.05
5		5×	6.22	6.32	+0.10
6		5×	5.54	5.64	+0.10
7		5×	5.64	5.69	+0.05
Average.....		5×	(7 samples.)		+0.08

TABLE VI.

Comparison of pH of Urines at 38° Determined Electrometrically on Undiluted Samples, and Colorimetrically on Samples Diluted Five Times.

Sample No.	Electrometric pH undiluted.	Colorimetric pH diluted five times (uncorrected).	Difference between electrometric and uncorrected colorimetric pH.	Difference between electrometric and corrected colorimetric pH.	Dye used.
1	5.11	5.28	+0.17	+0.07	Brom cresol green.
2	6.03	6.17	+0.14	+0.04	“ purple.
3	6.16	6.31	+0.15	+0.05	“ “
4	6.98	7.07	+0.09	-0.01	Phenol red.
5	6.83	6.92	+0.09	-0.01	“ “
6	6.34	6.39	+0.05	-0.05	Brom cresol purple.
7	6.35	6.43	+0.08	-0.02	“ “
8	4.51	4.64	+0.13	+0.03	“ green.
9	4.49	4.58	+0.09	-0.01	“ “
10	5.46	5.60	+0.14	+0.04	Chlor phenol red.
Average.....			+0.11	± 0.03	

one would not expect a constant correction in all urines at any one dilution, unless it were possible to dilute the urine with an

iso-ionic solution. However, one may subtract a correction of 0.1 pH for the effect of 5- to 10-fold dilution without introducing a significant error.

Comparison of Electrometric and Colorimetric Determinations of Urine pH.—Table VI gives electrometric and colorimetric determinations on urines diluted 5-fold after saturation with CO_2 and hydrogen as above. At this dilution, the correction involved in the colorimetric determination may be attributed almost entirely to the effect due to dilution. As one dilutes, the salt error of the dye decreases while the dilution error increases. At 5- and 10-fold dilution, the dilution error accounts for most of the total correction. The next to the last column gives corrected differences between the actual pH and the colorimetric determination. Although urines vary in their content of salts and other substances affecting the dye, and in their behavior on dilution, one may apparently subtract a correction of 0.1 pH from the colorimetrically observed pH in the 5- to 10-fold diluted urine at 38° , and be within 0.1 of the actual pH.

SUMMARY.

The apparent dissociation constants of brom cresol green, chlorophenol red, and brom cresol purple have been determined by the colorimetric method at 38° and 20° .

Using bicolor standards and an empirical correction for the error introduced by dilution of the urine, a technique for the colorimetric determination of urine pH is described.

A comparison of electrometric and colorimetric pH determinations in urine has been made.

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A MORE SPECIFIC REAGENT FOR THE DETERMINATION OF SUGAR IN URINE.

By JAMES B. SUMNER.

From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca.)

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The values given for the sugar of normal urine by the dinitrosalicylic method¹ recently described by the author are lowered by about 30 per cent if the urine is first purified by shaking with charcoal prepared according to the directions of Benedict and Osterberg.² The picric acid-acetone method of Benedict and Osterberg² when used with urine purified by charcoal gives results that are somewhat lower still than those given by dinitrosalicylic acid.

The reason why dinitrosalicylic acid gives results with normal urine that are too high is due, largely if not entirely, to the phenols present in the urine. Although phenol has no reducing action upon dinitrosalicylic acid it increases greatly the amount of color given by glucose. When an excess of phenol is present the amount of color given by 1 mg. of glucose is increased by 300 per cent. This color is of a different shade than the color given by a pure glucose solution, which fact causes the test with normal urine to make a poor match with the standard. When the dinitrosalicylic reagent has phenol added to it the color produced upon heating with glucose is unstable and changes in quality upon standing in contact with the air. This change can readily be prevented by the addition of a small quantity of sodium bisulfite. Accordingly, the dinitrosalicylic reagent is now prepared by adding to it both phenol and sodium bisulfite. The bisulfite doubtless aids also, as has been found by Benedict for his new

¹ Sumner, J. B., *J. Biol. Chem.*, 1924-25, lxii, 287.

² Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1921, xlviii, 51.

blood sugar method,³ in preventing somewhat the destruction of sugar by the alkali.

The new reagent is entirely satisfactory in every respect for the estimation of sugar both in normal and in diabetic urine. It gives an intense color with normal urine which matches the standards perfectly and which does not change in value for $\frac{1}{2}$ hour. The figures obtained are over 40 per cent lower than those given by the previously described reagent. This is a lower value than is given by the Benedict and Osterberg method even after using charcoal, and it seems certain that the new reagent is almost completely specific for reducing sugars. After shaking urine with charcoal according to the directions of Bene-

TABLE I.
Percentage Reduction of Normal Urine.

Urine No.	Old method.	New method.	
		Without charcoal.	With charcoal.
1	0.15	0.095	0.092
2	0.11	0.066	0.060
3	0.046	0.030	0.027
4	0.16	0.096	0.087
5	0.15	0.093	0.093
6	0.071	0.048	0.040
7	0.22	0.11	0.11
8	0.085	0.047	0.042
9	0.11	0.062	0.055
10	0.21	0.12	0.12

dict and Osterberg² the values given by the new reagent are reduced by about 6 per cent on the average, but this is due in part, at any rate, to the adsorption of some of the sugar by the charcoal. It was found that the charcoal adsorbed about 2 per cent from an acid sugar solution and 6 per cent from a neutral sugar solution when the concentration was 0.5 mg. of glucose per cc.

Table I shows how the new method compares with the old method and the effect upon the new method of using urine that has been treated with charcoal.

³ Benedict, S. R., *J. Biol. Chem.*, 1925, lxiv, 207.

It is not known how long the new reagent will keep, but it can be stated that it will last for over a month, if not longer. The time of heating is 5 minutes. Heating for 15 minutes increases the color production by only about 2 per cent. Doubling the amount of phenol added increases the production of color very little.

It may be of interest to note that fructose reduces the new reagent at room temperature much more rapidly than other reducing sugars, but that the sugar present in normal urine reduces the new reagent even more rapidly than fructose.

Preparation of the Reagent.

To 10 gm. of crystallized phenol add 22 cc. of 10 per cent sodium hydroxide. Dissolve in a little water and dilute to a volume of 100 cc. Weigh out 6.9 gm. of sodium bisulfite and add to this 69 cc. of alkaline phenol solution. Now add a solution containing 300 cc. of 4.5 per cent sodium hydroxide, 255 gm. of cochelle salt ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) and 880 cc. of 1 per cent nitrosalicylic acid solution. Mix and keep tightly stoppered in well filled bottles.

Method.

Pipette into a Folin-Wu sugar tube 1 cc. of urine (diluted if necessary) and 3 cc. of the reagent. Mix and heat 5 minutes in boiling water. Cool 3 minutes in running water, dilute to 5 cc. volume, mix, and compare in colorimeter with standard prepared with 1, 0.5, or 0.25 mg. of glucose, according to the concentration of sugar in the urine.

Interpretation of Results.

Concentrated urines, containing over 0.18 per cent of sugar, or dilute urines, containing over 0.12 per cent of sugar, can be considered abnormal.

THE CARBOHYDRATE METABOLISM OF TUMORS.

I. CHANGES IN THE SUGAR, LACTIC ACID, AND CO₂-COMBINING POWER OF BLOOD PASSING THROUGH A TUMOR.

BY CARL F. CORI AND GERTY T. CORI.

(From the State Institute for the Study of Malignant Disease, Buffalo.)

(Received for publication, May 13, 1925.)

It is the object of this paper to adduce further evidence that Warburg's experiments on tumor tissue *in vitro* are also valid for *in vivo* conditions. The work of Warburg, Poserer, and Negelein (1) has already been discussed in the first paper (2) of this series, where it was shown that certain experiments of Warburg on surviving tumor tissue could be duplicated on tumor-bearing animals. The following point seemed to require further investigation.

According to Warburg, tumor cells produce in 1 hour an amount of lactic acid that is equivalent to about 2 per cent of their fresh weight. Yet, tumors of starving animals were found to contain, as an average, only 0.034 per cent of lactic acid. How could this discrepancy be explained? Some experimental proof could be advanced that the low lactic acid content of the tumors was due to the fact that up to a certain limit an excess of lactic acid can be completely eliminated into the blood stream. It was thought that this question could be definitely settled by comparing the lactic acid content of blood that passed through a tumor with blood that has passed through normal tissues. The analysis of the systemic blood would be less favorable for the following reasons. An increase in the lactic acid content of the systemic blood could only be expected if the tumor constituted a large enough percentage of the body weight. The ability of the liver and the muscles to take up lactic acid from the blood is well known. Unless an amount of lactic acid constantly enters the blood, which surpasses the capacity of these organs to

398 Changes in Blood Passing through a Tumor

withdraw it, the lactic acid content of the blood will remain normal. It has been shown previously (2) that glucose administration raises the lactic acid content of the tumor to 0.100 to 0.180 per cent. Under these conditions, an increase of the lactic acid content of the systemic blood could be demonstrated in two cases, where the tumor constituted 13.7 and 22 per cent of the body weight respectively. Four analogous results have since been obtained. Due to the lack of suitable tumor material, the experiments are not numerous enough to warrant publication.

The first observations of the lactic acid content of blood that has passed through a tumor were made on a patient, who had a large sarcoma on the forearm. The vein, leaving the tumor, could easily be punctured. Since human tumors of such a favorable location are of a rare occurrence, it was found necessary to make the further experiments on animal tumors. Use was made of the Rous chicken sarcoma, which grows to a large size when transplanted into the musculature of the wing. By analyzing blood that was drawn from the normal wing and from the tumor-bearing wing, observations of the changes in the composition of blood passing through a tumor could be made.

EXPERIMENTAL.

Plymouth Rock chickens were used. The transplantations were made by injecting fresh emulsions of tumor cells in salt solution into the musculature of the fore limb. In about 3 weeks, the tumors were of the desired size of about a hen's egg. These tumors always showed a more or less necrotic area in the center.

The axillary veins of both sides were exposed and freed from the surrounding tissues shortly before the experiment was started and great care was taken to prevent any flapping of the wings. If the chickens did not remain perfectly quiet they were given a long period of rest before the blood was collected. Due to the position of the veins, blood could not be drawn simultaneously from both sides. Control experiments were therefore made, in which blood drawn alternately from the two sides was analyzed for sugar and lactic acid. If 2 to 3 cc. of blood were taken from each side, the time interval was about 2 to 3 minutes and

t made no marked difference from which side the blood was taken first. If 6 to 8 cc. of blood were drawn from each side, the time interval was longer and the sugar concentration of the blood that was drawn last tended to be a few mg. higher. The lactic acid concentration, however, remained uninfluenced. It seemed advisable to alternate the side from which the blood was taken first on the different chickens and this course was followed in our experiments. Generally, only 2.5 to 3 cc. of blood were drawn from each side, which sufficed for quadruplicate sugar and lactic acid determinations. If duplicate CO_2 analyses were intended, 6 cc. of blood had to be collected. In several experiments, in order to check the results of the first blood sampling, a small amount of blood for sugar determinations was taken, 1 or 2 hours later, in the reversed order. Precautions against glycolysis were not necessary, since the blood was analyzed as soon as it was drawn.

The method for blood sugar was that of Hagedorn and Jensen (3) which seemed well adapted for our purpose, since it allowed very accurate determinations with 0.1 cc. of blood. Since the blood corpuscles of chickens sediment extremely rapidly, special attention had to be paid to a thorough mixing of the blood. The lactic acid was determined by the Clausen (4) H_2SO_4 procedure. This method has already been used on former occasions and certain precautions, which are not described in Clausen's paper were found of importance for the outcome of the results. The concentrated sulfuric acid should be added to the solution containing the lactic acid very slowly and under constant cooling. Care should be taken that the two fluids do not mix before aeration is started. Generally, four determinations were run simultaneously. The aeration tubes leading into the lactic acid-sulfuric acid mixture should be of as small and as equal diameter as possible. The aeration should be maintained at a rather slow and very uniform rate. Compressed air was found to serve this purpose better than a suction pump. The decrease in the volume of the reaction mixture should not be too large and should be the same in all tubes. If an excess of iodine is used for the decomposition of the free bisulfite, the following titration of the bound isulfite gives too low results. The greater part of the unbound isulfite was removed with 0.1 N iodine, for the end-point, how-

400 Changes in Blood Passing through a Tumor

ever, 0.01 N iodine was carefully added until a faint blue to starch was obtained. The CO₂ analyses were made according to the technique of Van Slyke and Stadie (5), using the fine bore type of the Van Slyke apparatus. For the determination of the CO₂-combining power the plasma was saturated with alveolar air. The blood for the CO₂-content was collected under oil and the plasma was separated under the precautions outlined by Cullen (6).

TABLE I.

Comparison of the Sugar, Lactic Acid, and CO₂-Combining Power in the Blood of the Right and Left Wing Vein of Normal Chickens.

Chicken No.	Blood sugar.			Blood lactic acid.			CO ₂ -combining power of plasma.		
	Right vein.	Left vein.	Difference.	Right vein.	Left vein.	Difference.	Right vein.	Left vein.	Difference.
	mg.	mg.	mg.	mg.	mg.	mg.	vol. per cent	vol. per cent	vol. per cent
1	208	208	0	31.2	33.8	-2.6	42.0	41.2	+0.8
2	209	205	+4	45.4	47.2	-1.8	51.1	52.1	-1.0
3	230	240	-10	23.4	25.2	-1.8	52.3	53.0	-0.7
4	216	224	-8	42.5	44.6	-2.1	49.3	49.2	+0.1
5	234	227	+7				61.1	61.8	-0.7
5*	267	269	-2	26.8	27.6	-0.8	49.9	49.7	+0.2
5*	253	254	-1	25.5	22.8	+2.7	60.5	60.9	-0.4
6	241	239	+2	26.2	24.7	+1.5	57.4	56.8	+0.6
6	225	219	+6	32.8	29.4	+3.4			
7	233	234	-1	52.7	52.1	+0.6	54.8	55.7	-0.9
Average...	232	232	0	34.0	34.1	-0.1	53.1	53.4	-0.3

* After subcutaneous injection of glucose.

Table I shows the results that were obtained when the blood from the right and left wing vein of normal chickens was compared. The blood of the right vein contained, as an average, the same amount of sugar, 0.1 mg., less of lactic acid and showed after saturation with alveolar air, 0.3 volume per cent less of CO₂ than the blood of the left vein. If blood that had passed through a tumor was compared with normal venous blood, the results were entirely different (Table II). The blood from the tumor-bearing wing contained, as an average, 23 mg. less of sugar and 16.2 mg. more of lactic acid, and showed after saturation with alveolar air 3.7 volumes per cent less of CO₂ than the blood that was

rawn from the normal wing. In addition, the CO₂ content of blood drawn under oil has been determined (Table III). The blood from the tumor-bearing wing showed, as an average, a

TABLE II.

Comparison of Sugar, Lactic Acid, and CO₂-Combining Power in the Venous Blood of the Tumor-Bearing Wing and the Normal Wing.

Chicken No.	Blood sugar.			Blood lactic acid.			CO ₂ -combining power of plasma.		
	Tumor vein.	Normal vein.	Difference.	Tumor vein.	Normal vein.	Difference.	Tumor vein.	Normal vein.	Difference.
	mg.	mg.	mg.	mg.	mg.	mg.	vol. per cent	vol. per cent	vol. per cent
1	217	242	-25	55.1	46.1	+9			
1*	364	396	-32	73.3	40.8	+32.5			
1	253	280	-27						
1*	400	426	-26						
2	191	214	-23	62.9	40.7	+22.2			
2*	242	278	-36	51.4	29.2	+22.2			
2	201	234	-33				54.9	58.8	-3.9
2*	444	482	-38				52.3	58.2	-5.9
3	263	243	-20	50.1	47.0	+3.1	45.7	48.4	-2.7
4	218	221	-3	28.6	17.5	+11.1	39.1	43.5	-4.4
4*	234	274	-40	58.6	42.9	+15.7	40.8	43.8	-3.0
5†				94.3	78.5	+15.8	31.4	33.5	-2.1
6	224	255	-31	83.2	52.3	+30.9	34.3	40.1	-5.8
7*	275	288	-13						
7	198	198	0	34.2	13.8	+20.4	51.6	54.6	-3.0
7	240	250	-10	30.1	29.5	+0.6	51.7	53.2	-1.5
7	220	260	-40	54.1	38.5	+15.6	50.4	53.0	-2.6
7	240	259	-19						
7	240	272	-32	58.6	20.3	+38.3	34.2	37.6	-3.4
8	219	243	-24	35.3	24.6	+10.7	50.9	57.3	-6.4
8	255	296	-41	32.6	13.1	+19.5			
9*	293	315	-22	43.0	39.1	+3.9			
10	258	265	-7	57.4	54.5	+2.9			
10	251	278	-27						
Average ..	258	281	-23	53.1	36.9	+16.2	44.8	48.5	-3.7

* After subcutaneous injection of glucose.

† Chicken moribund. Died 1½ hours after blood sampling.

.7 volume per cent lower CO₂ content than the blood from the normal wing. The experiments on the patient referred to in the introduction are recorded in Table IV and show that the blood

402 Changes in Blood Passing through a Tumor

coming from a human tumor also contains less sugar and more lactic acid than blood that has passed through normal tissue. It should be noted that the tumor of this patient had been treated with x-rays 1 month previously. The response of the tumor to the treatment, however, was not very marked.

TABLE III.

Comparison of the CO₂ Content of Blood Drawn under Oil from the Tumor Vein and the Normal Vein.

Chicken No.	CO ₂ content of plasma.		
	Tumor vein.	Normal vein.	Difference.
	<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>
7	52.3	53.1	-0.8
7	51.7	52.8	-1.1
8	55.9	56.8	-0.9
8	55.2	56.1	-0.9
8	60.2	59.8	+0.4
Average.....	55.0	55.7	-0.7

TABLE IV.

Comparison of the Sugar and Lactic Acid Content in the Venous Blood from the Tumor-Bearing Arm and the Normal Arm of a Patient with a Sarcoma on the Forearm.

Blood sugar.			Blood lactic acid.			Remarks.
Tumor vein.	Normal vein.	Difference.	Tumor vein.	Normal vein.	Difference.	
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
90	101	-11	31.8	27.8	+4.0	Fasting.
248	257	-9	32.8	17.6	+15.2	1 hr. after 50 gm. glucose.
89	112	-23	23.9	17.4	+6.5	Fasting.
164	171	-7	32.0	18.9	+13.1	1 hr. after 50 gm. glucose.
198	207	-9				1 " " 50 " "
82	95	-13	27.9	24.1	+3.8	Fasting.
Average...145	157	-12	29.7	21.1	+8.6	

DISCUSSION.

The experiments in Table II support our previous conception that the lactic acid content of tumors remains low, because the excess that is produced in the tumors is eliminated into the

blood stream. The blood drawn from the vein of the tumor-bearing wing contained, in the majority of the cases, decidedly more lactic acid than the venous blood of the normal wing. One chicken, which is not recorded in Table II, was negative upon repeated examination. This could be traced to an almost complete necrosis of the tumor. The agreement of the lactic acid content of the venous blood from the right and left wing vein of normal chickens (Table I) shows that the method is accurate enough to regard even small differences in the lactic acid content as positive. The increase in the lactic acid content of the blood from the tumor vein was in most cases far beyond the error of the method.

The lactic acid in Clausen's method, as in other similar methods, is first converted into acetaldehyde. The latter is then distilled over into a sodium bisulfite solution. Yet, several other substances, as for instance acetone bodies, yield bisulfite compounds. There could have been such substances in the blood that has passed through a tumor, which would have been determined as lactic acid because of their bisulfite-binding properties. The solution of the lactic acid as the zinc salt did not seem an accurate enough procedure with the small amounts of blood available. It was argued that if more lactic acid entered the blood on the tumor side than on the normal side differences in the CO_2 -combining power of the plasma should be detected, since the excess of acid on the tumor side would bind part of the available base. Table II shows that the plasma from the tumor side, after saturation with alveolar air, yielded as an average, 3.7 volumes per cent less of CO_2 than the plasma from the normal side. This was not due to a difference in the CO_2 content, since blood collected under oil and centrifuged under the necessary precautions showed only 0.7 volume per cent less of CO_2 in the plasma from the tumor side than from the normal side, as is illustrated in Table IV. It is, therefore, demonstrated by an independent, though indirect method, that more acid is contained in the blood that has passed through a tumor than in blood that has passed through normal tissue. A calculation will show that the agreement between the two methods is satisfactory.¹ The experiments

¹ It was found in several experiments with chicken's blood that lactic acid determinations in whole blood and in plasma gave almost identical values.

404 Changes in Blood Passing through a Tumor

in Table II, in which both the lactic acid and the CO₂-combining power have been determined, show that the blood of the tumor vein contained, as an average, 16.3 mg. more of lactic acid and 3.5 volumes per cent less of CO₂ than the blood of the normal vein. 1 molecule of lactic acid displaces 1 molecule of CO₂. 3.5 cc. of CO₂ at 0°C. and 760 B. weigh 6.87 mg. and correspond to $\frac{90.1 \times 6.87}{44}$ or 14.0 mg. of lactic acid. It can, therefore, be concluded that most of the lactic acid that leaves the tumor is in the free state.

The strong glycolysis that is taking place in the tumor tissue sets up a constant demand for sugar. This explains why the blood sugar in the tumor vein was decidedly lower than in the normal vein. In three experiments in Table II, the difference was not very marked. In two of these cases, a rather large amount of blood was drawn in order to include CO₂-combining and CO₂ content determinations and the blood from the tumor side was drawn last. We are able to confirm the previous observations of Tadenuma, Hotta, and Homma (7), who noted a diminution of the sugar content of the blood drawn from the vein of a tumor-bearing wing. Other substances than sugar were not determined by these authors.

The observations that were made on the chicken sarcoma could be duplicated on a human tumor (Table IV). This seems of importance since the chicken sarcoma has a unique position among all other malignant tumors. It is in all probability caused by a filtrable virus. Warburg (8) mentioned in a recent communication that the chicken sarcoma showed a glycolysis of a similar magnitude to that of other malignant tumors. The results recorded in this paper are valid for malignant tumors in general and they are another confirmation of Warburg's *in vitro* experiments under *in vivo* conditions.

SUMMARY.

1. On comparing the sugar and lactic acid contents and the CO₂-combining power of the plasma of blood drawn from the right and left wing vein of normal chickens, identical values were obtained.

2. If a tumor was growing on one wing, the blood that had passed through the tumor contained, as an average, 23 mg. less of sugar and 16.2 mg. more of lactic acid than the blood that had passed through the tissues of the normal wing.

3. The plasma obtained from the blood of the tumor side showed upon saturation with alveolar air 3.7 volumes per cent less of CO_2 than that of the normal side. The plasma of blood that had been collected under oil contained as an average only 0.7 volume per cent less of CO_2 when compared with that of the normal side.

We are under obligation to Dr. James B. Murphy from The Rockefeller Institute, who kindly supplied us with the chicken sarcoma powder.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM.

III. DEDUCTIONS CONCERNING THE CAPILLARY EXCHANGE.

BY L. J. HENDERSON AND C. D. MURRAY.

(From the Claude Bernard Laboratory, Névache, France.)

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The present paper is devoted to a discussion of deductions which may be drawn, from the physicochemical properties of blood, concerning the capillary exchanges of oxygen and carbonic acid, the area of the capillary bed, and the circulation of blood.

In the preceding paper of this series (1) it is demonstrated¹ that a graphical integration leads directly, from a suitable description of its physicochemical properties, to a definition of the sequence of changes of the blood which constitute the respiratory cycle. Experimental data apart, this procedure rests upon no assumption except that the entrance and exit of oxygen and carbonic acid are phenomena of diffusion, not measurably complicated by unknown phenomena. The accuracy of the method is somewhat uncertain because, among other things, the coefficients of diffusion of oxygen and carbonic acid within the body are not accurately known, but there is little reason to doubt the substantial trustworthiness of the result. The immediate outcome of this integration is represented by Fig. 1.

Here the sequence of simultaneous changes in the concentrations of free and combined carbonic acid and oxygen, as the blood passes through the capillaries, is represented graphically by a cycle. From these all other changes may be deduced with the aid of the large nomogram of the preceding paper. This description applies to that case only when the composition of arterial blood and that of venous blood are represented by the points A and V. For any other pair of arterial and venous points integration will yield another cycle.

¹ Henderson, Bock, Field, and Stoddard (1), Section III, pp. 424-428.

As briefly indicated in the preceding paper, it is possible to proceed to further results. One of these has already been set forth in discussing the rate of change of blood in its movements through capillaries;² it is now possible to present several others.

In order to fix our ideas we may begin with a tabulation of simultaneous values of the concentrations of free oxygen and oxyhemoglobin, throughout the cycle represented by Fig. 1.

Taking first the case of the lung, it is evident that Table I makes possible the calculation, for every value of HbO_2 , of the effective

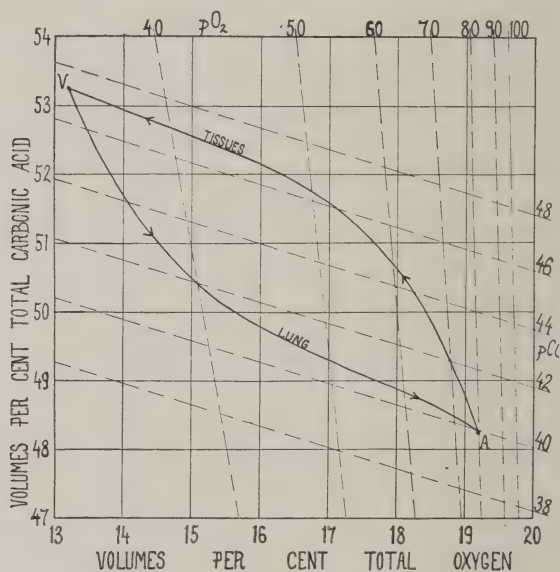


FIG. 1.

head of oxygen pressure causing the diffusion of oxygen from alveolar air to blood. This head of pressure is, in fact, equal to the mean partial pressure of oxygen in the alveolar air, minus the concentration of free oxygen in blood, expressed as mm. of mercury. Thus it is easy by simple subtraction, given the partial pressure of oxygen in the alveolar air of A.V.B. as 110 mm., to obtain the values of Table II.

² Henderson, Bock, Field, and Stoddard (1), p. 428.

Making use of this table, we may next lay off as ordinates on fig. 2 values of HbO_2 , and at convenient intervals draw sets of parallel lines whose slopes measure the head of oxygen pressure or the ordinates on which they are placed. Then that curve which, beginning at $\text{HbO}_2 = 66$ per cent and ending at $\text{HbO}_2 = 6$ per cent, is everywhere parallel, at the corresponding values of

TABLE I.

HbO ₂ saturation.	$p\text{O}_2$	
	Lung.	Tissues.
<i>per cent</i>	<i>mm.</i>	<i>mm.</i>
65	34.0	34.0
70	37.0	37.4
75	40.5	41.3
80	44.7	45.7
85	50.0	51.4
90	58.0	61.2
95	73.2	75.0
96	78.0	78.0

TABLE II.

Lung.

HbO ₂ saturation.	O ₂ head (Δp).
<i>per cent</i>	<i>mm.</i>
65	76.0
70	73.0
75	69.5
80	65.3
85	60.0
90	52.0
95	36.8
96	32.0

bO_2 , with the slopes thus drawn, represents the necessary course of the diffusion process in the lung. Therefore, if we assume uniformity of structure and of blood flow in the capillary, the abscissa under the curve may be taken to represent the length of an average lung capillary, or, what comes to the same thing, time.

This may be stated mathematically in very simple form. Let

R be rate of diffusion; Δp , head of oxygen pressure; s , quantity of oxygen diffusing; and t , time. Then

$$R = k\Delta p = \frac{ds}{dt}$$

Accordingly, it is only necessary to divide the abscissa under the curve into ten equal parts and to read from the curve the value

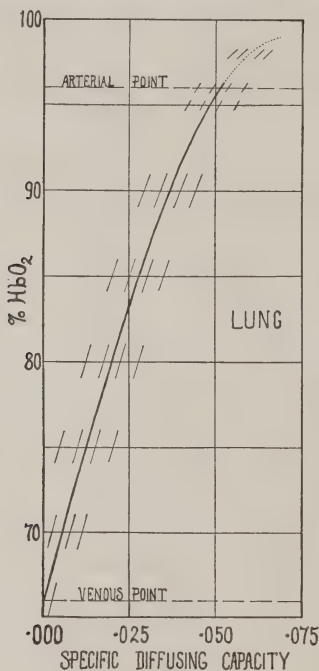


FIG. 2.

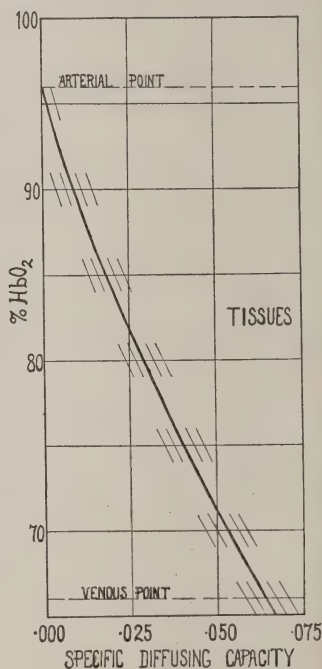


FIG. 3.

of HbO₂ corresponding to these divisions, in order to be able to graduate the process of diffusion in the lung in the manner of the preceding paper.

On the assumption that the oxygen concentration in the tissue is negligibly small, and taking into consideration an average capillary, which delivers blood of the composition of mixed venous blood to the vein, it is easy to repeat the construction and thus to obtain a corresponding result for a capillary of the greater circula-

lation. This has been done in Fig. 3. Here also one is dealing with an ideal capillary corresponding to a statistical mean, and in this case, no doubt, the extreme departures from the mean which actually occur in the organism are very large. It is also extremely improbable that in all parts of the body the oxygen concentration of the tissues should be negligibly small. We shall return to a consideration of these difficulties.

From what has been said above it is evident that we may draw useful conclusions from a comparison of the lengths of the ab-

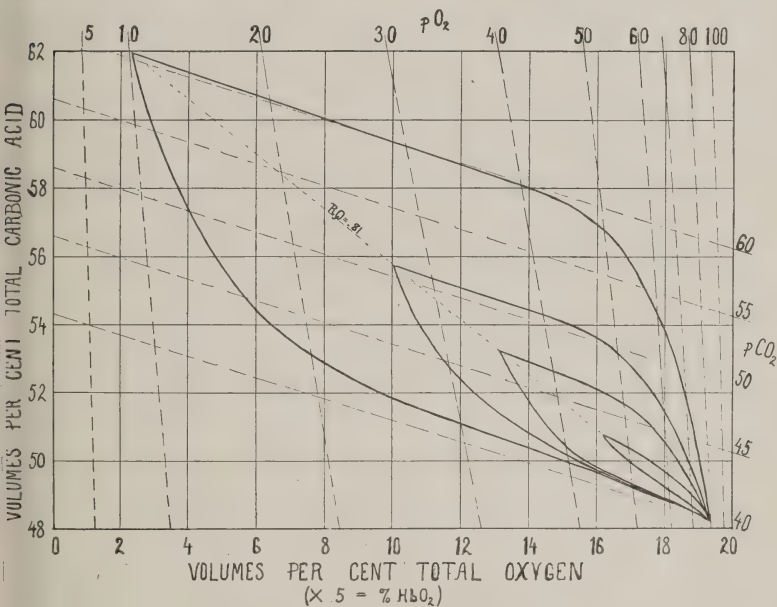


FIG. 4.

scissæ under the curves of Figs. 2 and 3. These lengths are, in fact, proportional to the total areas of uniform diffusing surface over which, in equal periods of time, equal volumes of arterial and venous blood must pass, under the conditions which have been assumed in the construction of the figures, in order that the respiratory exchange may be accomplished. Each length may be regarded as a measure of the *specific diffusing capacity* of the capillary system in question, in other words of the diffusing capacity, per liter of blood flow, per minute, of the capillaries of the lesser

and of the greater circulations respectively. Their ratio measures, therefore, the relative diffusing capacities of lung and tissue capillaries for the conditions now under discussion. We may draw the conclusion that in a normal individual, under ordinary conditions which will later be shown to exist during moderate exercise, when about one-third of its oxygen content is removed from the

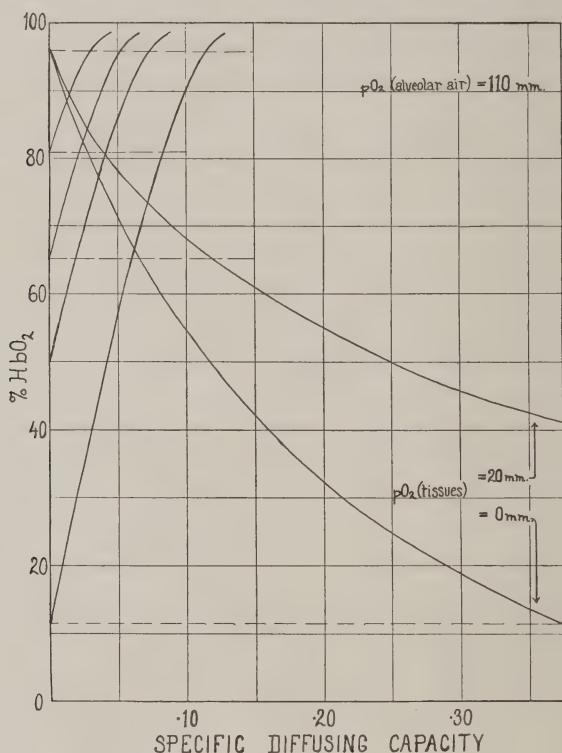


FIG. 5.

blood in its passage through the greater circulation, the diffusing capacity of the active capillaries of the greater circulation must be at least 20 per cent greater than that of the active capillaries of the lung.

There is no difficulty in repeating this investigation for other cases. At present it will perhaps suffice to restrict our attention to three other instances in which the arterial point as well as the respiratory quotient remain unchanged, while the venous point

falls at 81, 50, and 11 per cent of HbO_2 , respectively. Fig. 4 gives the result of the first integration for these three cases, and also for the original case. Once more the oxygen of the alveolar air is assumed to be at a pressure of 110 mm., that in the tissues at 0 mm.

In Fig. 5 the results of the second integration for all four cycles are represented. In addition there is given the result obtained on the assumption that the pressure of oxygen in the tissues of the greater circulation is 20 mm. From this figure it is possible to read the values of the specific diffusing capacity for eleven cases. These are assembled in Table III.

TABLE III.

Specific Diffusing Capacity (Arterial Point = 96 Per Cent of HbO_2).

Venous point, per cent.....	81	66	50	11
Lung ($p\text{O}_2 = 110$).....	0.030	0.051	0.072	0.114
Tissues ($p\text{O}_2 = 0$).....	0.027	0.063	0.118	0.375
“ ($p\text{O}_2 = 20$).....	0.038	0.120	0.250	

TABLE IV.

Diffusing Capacity per Liter of Oxygen per Minute (Arterial Point = 96 Per Cent of HbO_2).

Venous point, per cent.....	81	66	50	11
ΔO_2 , per cent.....	15	30	46	85
Lungs ($p\text{O}_2 = 110$).....	1.00	0.85	0.79	0.67
Tissues ($p\text{O}_2 = 0$).....	0.90	1.05	1.29	2.21
“ ($p\text{O}_2 = 20$).....	1.27	2.00	2.74	

More clearly to illustrate the conditions imposed upon the organism by the properties of the blood, the data of Table III have been converted into those of Table IV. Here are presented the values of diffusing capacity, per liter of oxygen diffusing, per minute.

These tables demonstrate that the total area of diffusing surface, which we may assume to be roughly proportional, for similar structures, to the number of physiologically active capillaries, is subject to wide variation. Such variation is the expression of a simple physical necessity. We are, fortunately, able to present a rough estimate of the magnitude of this variation in one instance.

In a series of experiments recently performed at the Massachusetts General Hospital, the blood of A. V. B., which has provided the data for all the above calculations, was found to be about 75 per cent saturated on its return to the heart during rest, and about 66 per cent saturated during moderate exercise on a stationary bicycle. The arterial blood meanwhile remained nearly unchanged at the value $\text{HbO}_2 = 96$ per cent. This exercise produced approximately a fourfold increase in the flow of blood per minute.

These conditions correspond to the following values of specific diffusing capacity.

	Rest.	Work.
Pulmonary circulation.....	0.040	0.051
Greater circulation.....	0.040	0.063

In order to obtain values of the total diffusing capacity, these values must be multiplied by the volume of blood flow per minute. Thus, taking the blood flow at rest as 5 liters per minute, the total diffusing capacity appears to have, in arbitrary units, the following values.

	Rest.	Work.
Pulmonary circulation.....	0.20	1.02
Greater circulation.....	0.20	1.26

It seems clear that really hard work must be accompanied by a further large increase of total diffusing capacity. Even moderate work, however, produces changes which are very striking. There seems no reason to doubt that the number of patent capillaries in the lung has increased in this experiment about fivefold, and unless there has been a substantial fall in the tension of oxygen in the muscles, the number of patent capillaries in the greater circulation must have undergone a still larger increase.

The increased transport of oxygen has been accomplished in this case by what appears to be an increase in the diffusing capacity of the capillary system approximately proportional to the increase in the total oxygen consumption, by a large but slightly smaller increase in the flow of blood, and by a small increase in

the coefficient of utilization of oxygen. Indeed it seems not impossible that in the active tissues the last of these variables has remained nearly constant. For we should expect that the blood returning from inactive parts of the body must contain more oxygen than that which has supplied active tissues, and it is clear that a larger fraction of the circulating blood passes through active tissues during exercise than during rest. Under these circumstances, an increase of activity might well bring about a substantial increase in the coefficient of utilization of oxygen for the whole body, although the coefficient for each organ and tissue remained substantially unchanged. Suppose, for example, that during rest 4 liters of the blood flow through inactive tissues which reduce the oxyhemoglobin to 78 per cent and 1 liter flows through active tissues which reduce the oxyhemoglobin to 63 per cent. If, then, during exercise the whole increase of blood flow, 15 additional liters, went to supply active tissues, and the coefficient of utilization of active and inactive tissues remained unchanged, the result would be in agreement with the observations above reported. No doubt the real phenomenon is very far from this simple illustration, which is intended merely to define one element of a complex adjustment.

On more general grounds, there is reason to suppose that the capillary blood of a particular tissue is likely to remain relatively constant in composition, preserving more or less precisely its characteristic gradient from the beginning to the end of the capillary, at least during moderate changes of activity. Indeed, ever since the hypothesis of the constancy of the internal environment was first stated by Claude Bernard (2), there has been little but confirmation of his views, which find particularly strong support in recent physicochemical discoveries.

However this may be, it is clear, as stated above, that the greatly increased call for oxygen has been met in this instance by a very large increase in the capillary bed and in the volume of the blood flow, accompanied by no great change in the amount of reduction of the blood in its passage through the active regions of the body. We may now inquire what other changes might supply an equal amount of oxygen. The answer to this question may readily be found with the help of Table IV. According to this table, the diffusing capacity necessary to permit the diffusion of

a given amount of oxygen into the lung is less, the greater the coefficient of utilization. This must always be true if the arterial point remains substantially unchanged. In the tissues, the greater the coefficient of utilization, the greater is the diffusing capacity required for a given quantity of oxygen.³ Thus it is apparent that it is ordinarily the conditions of diffusion in the tissues of the greater circulation, and, therefore, particularly in the muscles which tend to associate a high degree of activity with a normal coefficient of utilization of oxygen, and to bring about the adjustment above described. For example, in order that in the tissue arterial blood of 96 per cent of HbO_2 may be reduced to venous blood of 11 per cent of HbO_2 , a diffusing capacity per liter of oxygen per minute of 2.21, instead of 1.05 for venous blood of 66 per cent of HbO_2 , would be required. This would lead, in the case of moderate exercise, to an increase of total diffusing capacity from the original value of 0.20 not merely to 1.26, but to 2.65. It is true that the blood flow would, under these circumstances, be increased only about 50 per cent (instead of fourfold) above the resting value. But this might be a very slight compensation. Indeed it might well be the reverse, for the blood would, under these circumstances, be moving with only about one-tenth its normal velocity along each capillary, a condition on many other grounds hardly compatible with great activity.⁴

Very different is the actual phenomenon, for in reality the blood is moving through each capillary, not only nearly unchanged in composition from its resting state, but also with nearly unchanged velocity, compared with the condition of rest.

This, in fact, is the concrete result of the present inquiry, that a considerable increase in activity of an organ may be accompanied by the opening of capillaries approximately in the same proportion and by a like increase in the total flow of blood. Such a readjustment can involve but little change in the blood flow per capillary and therefore in the composition of the blood and in the physicochemical conditions of the environment of the cells.

In the light of these considerations the opening and closing of

³ With constant venous point and varying arterial points, these tendencies are reversed.

⁴ For a full exposition of these and other related questions, the following paper should be consulted.

capillaries described by Krogh (3) appears as one of the major physiological activities.

SUMMARY.

In this paper the diffusing capacity of the capillary bed has been estimated as a deduction from the physicochemical properties of blood, the composition of arterial and venous bloods, and the volume of the blood flow.

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OXYGEN EXCHANGE, BLOOD, AND THE CIRCULATION.

A COORDINATED TREATMENT OF THE FACTORS INVOLVED IN OXYGEN SUPPLY ON THE BASIS OF THE DIFFUSION THEORY.

BY CECIL D. MURRAY AND WILLIAM O. P. MORGAN.

(From the Physiological Laboratory, Cambridge University, England.)

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In the transport of oxygen from lungs to tissues and in the local processes of oxygen exchange, several anatomical and functional components are involved: lungs, the heart and larger blood vessels, capillaries, and blood itself. It will be realized that the preceding paper (1) deals with two types of interconnections existing among these components. The first type includes relations which are governed, as it were, by well established physicochemical laws conditioned by the known properties of the various elements of the system. This set of relations constitutes the quantitative physicochemical background of the process of oxygen exchange. As will appear later, this process can be described with very fair accuracy as the steady operation of a system with six degrees of freedom. The second type includes relations governed by physiological laws, the nature of which are only vaguely perceived, but which are recognized by the further restrictions, perhaps statistically defined, imposed upon the wide physicochemical scheme. These laws not only delimit a physiological "range" upon the general background, but they also determine a correlation between variables which otherwise, from the purely physical point of view, are apparently independent. The distinction between the two types of relations is perhaps only a temporary necessity, but it at least suggests and emphasizes a dominant problem.

The phenomenon of adaptation can well be studied in connection with a quantitative and detailed description of a particular and simple physiological function, which, though complex in its manner of operation, is yet thoroughly investigated. With this

in mind we have not thought it superfluous to elaborate into a scheme those relations, discussed in the preceding paper, which are of a purely physical and chemical nature. The scheme to be presented includes a few additional variables and describes as far as possible the whole process of oxygen transport in a generalized and coordinated manner. Whatever may have developed which contributes to purely physiological problems will not be referred to in this paper, for it is our immediate purpose to describe the physically possible variations in the process of oxygen exchange which are available to man and to animals with blood and circulatory systems of the human type. There can follow a study of those variations or particular paths which are actually "chosen" by the organism.

Description of the Oxygen System.

Of the factors involved in the "oxygen function" of the organism many can be dealt with analytically, some however cannot, and therefore, for the time being, the basis of any coordinated treatment must be a graphical one. We can begin at once by referring to Fig. 2 of the preceding paper (1). From the method of its construction, it will be noticed that, if a straight line be drawn on this figure from the venous point to the arterial point, it will have a slope which defines a certain hypothetical, "average" head of oxygen pressure between alveolar air and blood of a composition varying from venous to arterial. This "mean" head of pressure (which can be designated $\Delta p'$, in contradistinction to Δp , the head at any particular point in time or along the length of a capillary) is *equivalent* to the sum of the heads of pressure at each small interval, multiplied by the fraction of the whole time or length represented by each small interval of time or length. In mathematical form

$$\Delta p' \times t = \int_{t_0}^{t_1} \Delta p dt$$

The factor $\Delta p'$ has been calculated for certain special cases by Bohr (2) and by Barcroft (3). It is introduced here because it is one of the two important factors which directly determine oxygen exchange across the capillary wall. The other factor is

also a complex one which depends on the capillary surface area, its thickness and the permeability to oxygen of the capillary wall, the temperature, etc. This second factor has likewise received some attention in the past, and has been discussed in Barcroft's report just alluded to. We will adhere, for the present discussion, to the term diffusing capacity, DC , which was used in the preceding paper. The diffusing capacity may be conveniently thought of as being proportional to the active capillary surface, hence, as suggested by the work of Krogh (4), physiological variations in this factor are especially significant.

According to a simple application of the law of diffusion

$$\Delta p' \times DC = MR \times k \quad (1)$$

where MR is the amount per minute of oxygen, measured in cc. of dry gas under standard conditions, which passes across a capillary wall of diffusing capacity, DC . If $\Delta p'$ is measured in mm. of mercury and if k is taken as one, $k = 1$, the unit¹ of DC is thereby arbitrarily defined.

It is also desirable to introduce the oxygen capacity of blood as a variable to be considered. Let Hgb , therefore, refer to the oxygen capacity of the blood in volumes per cent; *i.e.*, to the cc. of O_2 (standard) contained in 100 cc. of blood in equilibrium with partial pressure of oxygen, $pO_2 = 145$ mm. With this additional variable the term specific diffusing capacity, SDC , must be extended to mean: the diffusing capacity of a given capillary region, per liter of flow per minute, BF , of blood of a *certain total oxygen capacity*, Hgb . The definition and the units² are implicit in the following equation.

$$SDC = \frac{DC}{BF \times Hgb} \quad (2)$$

The term *specific* diffusing capacity emphasizes the adjustment of diffusing capacity to the product $BF \times Hgb$ —a product we

¹ The unit of DC is here twenty times greater than the unit for diffusing capacity as calculated in the preceding paper (1).

² The unit of SDC is here the same as that used in the preceding paper. It will be seen that the introduction of the factor Hgb (which had the value .20 in the case discussed in the previous paper) has necessitated a corresponding change in the unit of DC .

422 Oxygen Exchange, Blood, and Circulation

may designate by the name hemoglobin flow and the abbreviation *HF*. The reciprocal of the specific diffusing capacity may be called the *specific* hemoglobin flow, *SHF*, thus emphasizing the adjustment of hemoglobin flow to diffusing capacity. Thus we can write

$$SHF = \frac{1}{SDC} = \frac{BF \times Hgb}{DC} = \frac{HF}{DC}$$

The slight amplifications of what is implicitly contained in Fig. 2 of the preceding paper (1) have now been defined, and will be obvious that similar reasoning applies equally to Figs. 3 and 5 of that paper. The purpose of the present additions will be more apparent later. A discussion of some underlying assumptions and simplifications is also reserved for the end.

In order to provide data which can be applied to all ordinary physiological conditions, data which will yield immediately the desired quantitative relations for any observed case, we have made fifteen constructions similar to Fig. 2, and six similar to Fig. 3 of the preceding paper. Among these twenty-one preliminary constructions (not reproduced here) are three sets, each set corresponding to a certain oxygen dissociation curve. The three curves used are chosen from those published by Bock, Field, and Adair (5), and further treated in the paper by Henderson, Bock, Field, and Stoddard (6). Their curves are given under the headings $p\text{CO}_2 = 80, 40, \text{ and } 20 \text{ mm.}$, indicating that in the determination of each curve the partial pressure of carbon dioxide was kept constant at the value stated. For the purpose of generalizing these curves, however, it is necessary, though perhaps not altogether sufficient, to define them by the hydrogen ion activity of the serum rather than by the carbon dioxide pressure. This point will be referred to again. Actually we have, as it were, relabelled these curves as corresponding sufficiently closely to curves for $\text{pH}_s = 7.25, 7.45, \text{ and } 7.65$ —values obtainable from the two papers cited.

With each of the three selected oxygen dissociation curves we have made constructions to cover the conditions where the pressure of oxygen in alveolar air assumes the values: $p\text{O}_2 \text{ alveolar} = 110, 100, 90, 70, \text{ and } 50 \text{ mm.}$, and where the oxygen pressure of tissues assumes the values: $p\text{O}_2 \text{ tissues} = 0 \text{ and } 20 \text{ mm.}$ The

we have chosen twenty-one conditions, and for each has been calculated series of values to cover practically all combinations of arterial and venous points. Fifteen of the constructed curves begin at a venous point where V per cent $\doteq 0$, and six begin at an arterial point, A per cent $= 100$. From one such curve—for instance for the case: pO_2 alveolar air $= 110$, $pH_s = 7.45$, and V per cent $= 0$ —values for $\Delta p'$ are calculated by dividing the difference in percentage saturation between arterial and mixed venous bloods, ΔO_2 per cent, for successively chosen arterial points, by the value of SDC as determined graphically at these points. The scale is selected so that

$$10 \Delta p' \times SDC = \Delta O_2 \%, \text{ or } 10 \Delta p' = \Delta O_2 \% \times SHF \quad (4)$$

Calculations for V per cent $= 10$ (and each subsequent venous point) with successive arterial points are then easily made with the aid of the tabulated data obtained from the curve beginning at V per cent $= 0$. Data thus obtained from one construction, including values of $\Delta p'$ and ΔO_2 per cent for combinations of arterial and venous points, can then be recast in graphic form yielding a chart, the rectangular coordinates of which measure $\Delta p'$ and ΔO_2 per cent. Twenty-one of these charts (the term chart will now be used to refer to one of this series) designated by Roman numerals, and arranged according to the following scheme, are presented here.

pH_s	Lung charts. pO_2 alveolar air.					Tissue charts. pO_2 tissues.	
	110	100	90	70	50	0	20
7.25	I	II	III	IV	V	VI	VII
7.45	VIII	IX	X	XI	XII	XIII	XIV
7.65	XV	XVI	XVII	XVIII	XIX	XX	XXI

The scale for $\Delta p'$, in mm. of mercury, is numbered at the top of each chart. ΔO_2 per cent, the scale of ordinates, is measured downward. On each of the lung charts is a set of curves, numbered at their lower extremities according to the arterial percentage saturations, A per cent which they represent. These lines are crossed by a few venous lines, and limited by the curve V per cent $= 0$. The limit of arterial saturation is given by the num-

424 Oxygen Exchange, Blood, and Circulation

bers in brackets (near the numeral designating the number of each chart), and this limit is properly represented by the vertical line, $\Delta p' = 0$. On the tissue charts the general positions of the arterial and venous lines are reversed, and the limit of reduction of the blood is given as before in brackets, which limit also is represented by the line $\Delta p' = 0$.

It will be noticed that, since ΔO_2 per cent is determined by part of the coordinate system, the set of arterial lines necessarily determines the set of venous lines on the same chart, and *vice versa*. The lines denoting specific hemoglobin flow, *SHF*, could have been drawn on these charts in accordance with Equation 4.

An example of the method of using the charts is as follows: Given pO_2 alveolar air = 95, $pH_s = 7.35$, A per cent = 95, and V per cent = 65; to find $\Delta p'$ and *SHF* by double interpolation. First, reference is made to Chart II ($pO_2 = 100$ and $pH_s = 7.25$), and here the intersection of the line ΔO_2 per cent = 30 with the curve A per cent = 95 yields the value $\Delta p' = 39$. Similarly, readings are made on three other charts as indicated in the following tabulation.

pH_s	$pO_2 = 100$	$pO_2 = 90$	$pO_2 = 95$
7.25	II : 39	III : 26	32.5
7.45	IX : 51	X : 40	45.5
7.35	45	33	39.0

The required value for $\Delta p'$ is 39 mm.; and $SHF = \frac{(39 \times 10)}{30} = 13$.

The choice of ΔO_2 per cent and $\Delta p'$ as the variables to be represented by the rectangular coordinates was made largely because of the simplicity of the following relations, the first two of which have already been stated.

$$\Delta p' \times DC = MR \quad (1)$$

$$10 \Delta p' = SHF \times \Delta O_2 \% \quad (4)$$

$$\Delta O_2 \% \times Hgb = 10,000 \Delta O_2 \text{ cc.} \quad (5)$$

$$1000 \Delta O_2 \text{ cc.} \times BF = MR \quad (6)$$

Equation 1 follows from the diffusion theory; the constant was taken to be one, and is omitted here. Equation 4 follows from

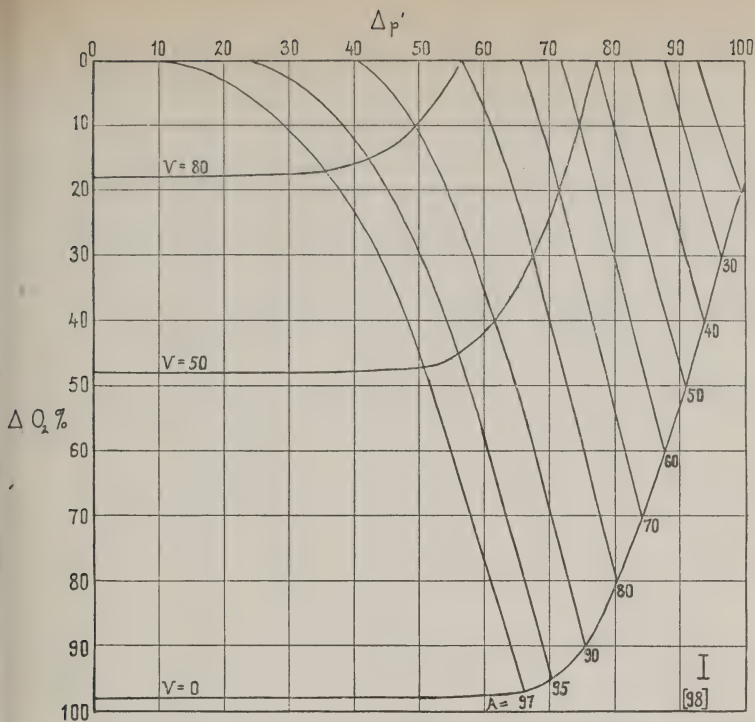


CHART I. pO_2 alveolar air = 110 mm.; $pH_s = 7.25$.

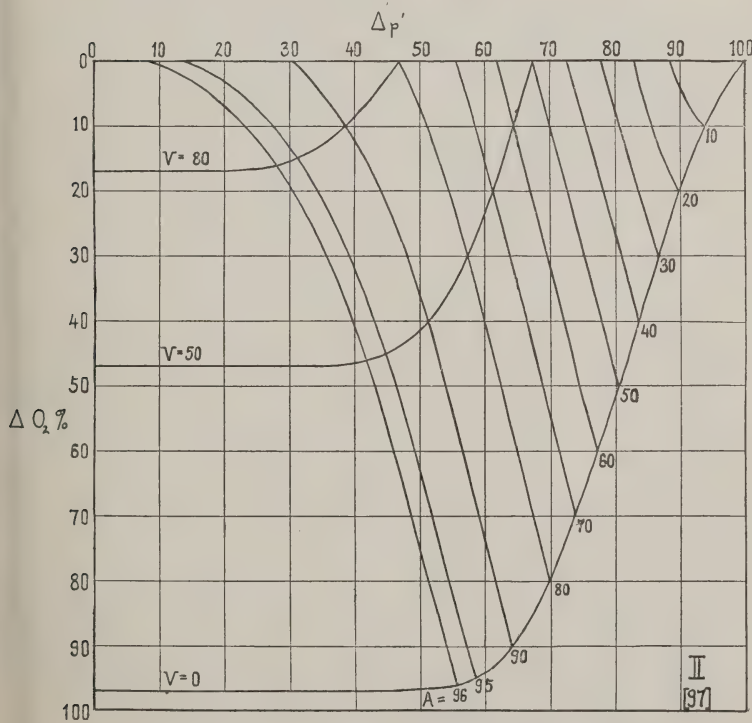


CHART II. pO_2 alveolar air = 100 mm.; $pH_s = 7.25$.

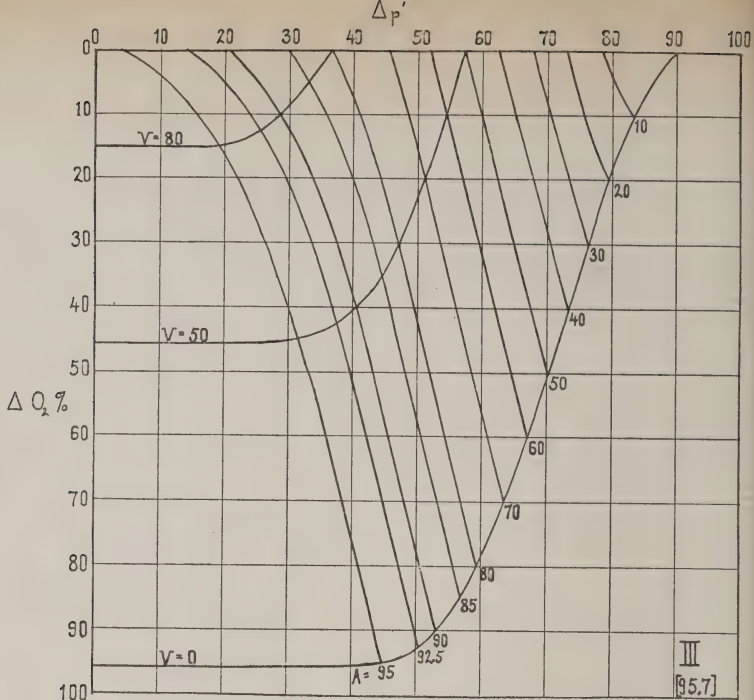


CHART III. pO_2 alveolar air = 90 mm.; $pH_s = 7.25$.

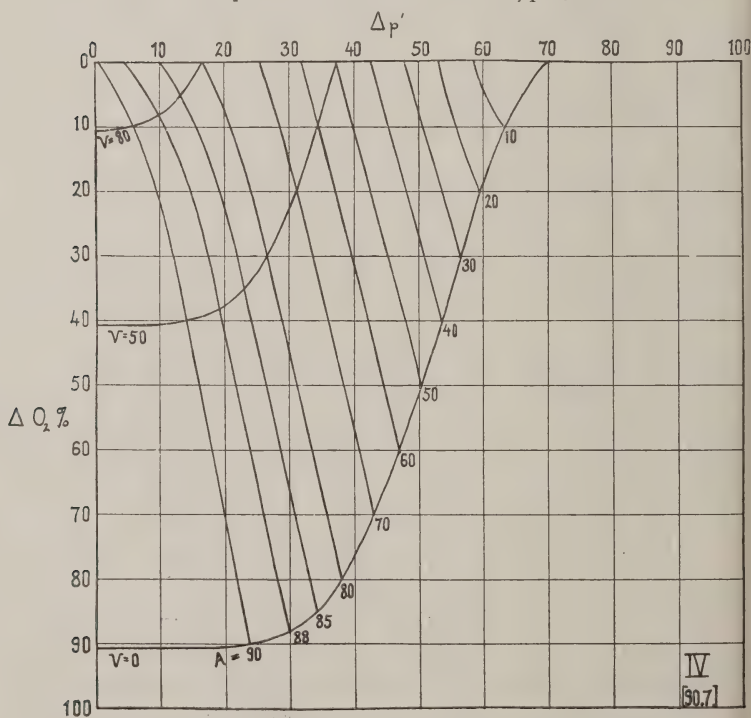


CHART IV. pO_2 alveolar air = 70 mm.; $pH_s = 7.25$.

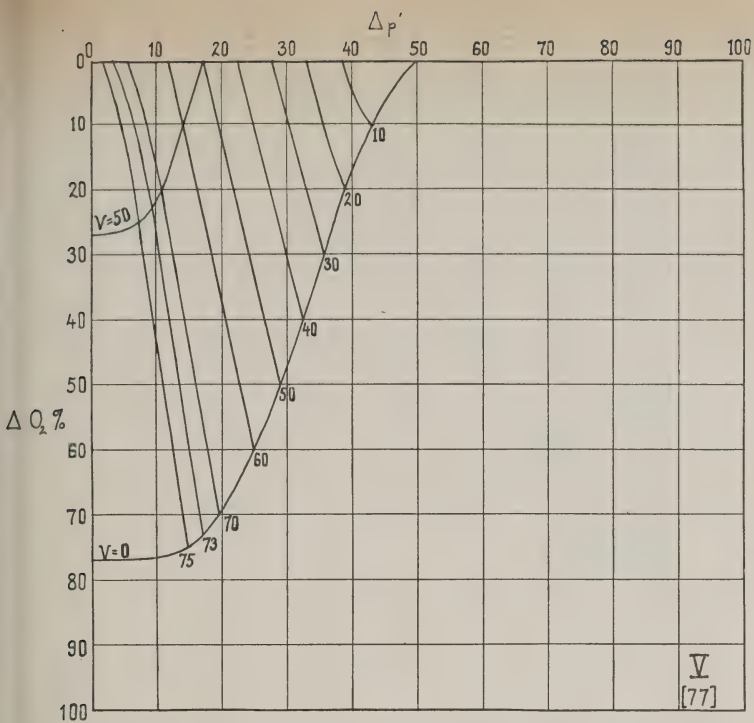


CHART V. pO_2 alveolar air = 50 mm.; $pH_s = 7.25$.

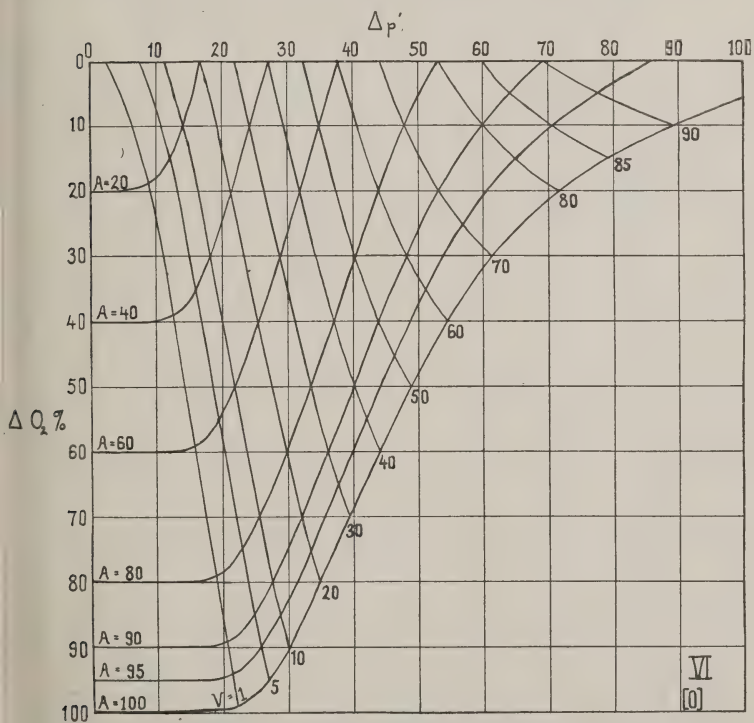


CHART VI. pO_2 tissues = 0 mm.; $pH_s = 7.25$.

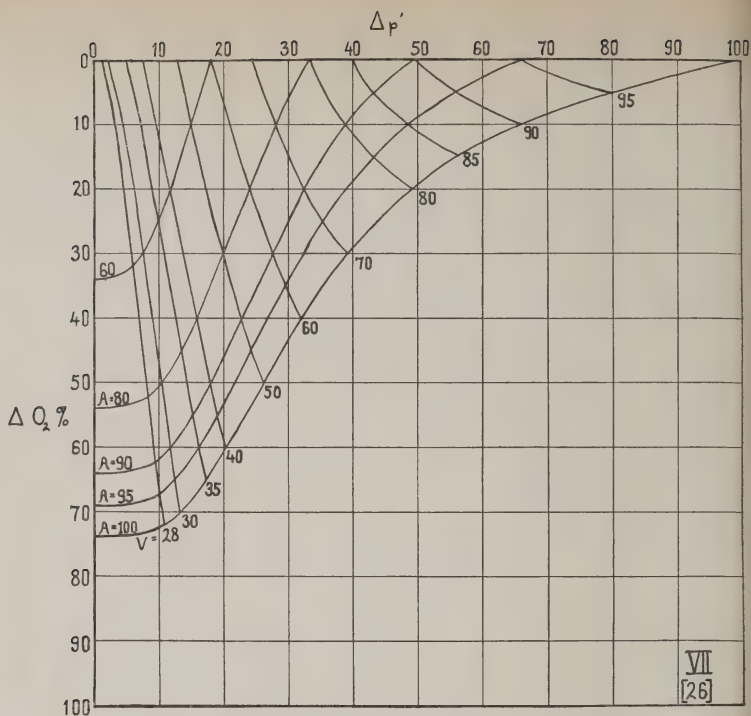


CHART VII. pO_2 tissues = 20 mm.; $pH_s = 7.25$.

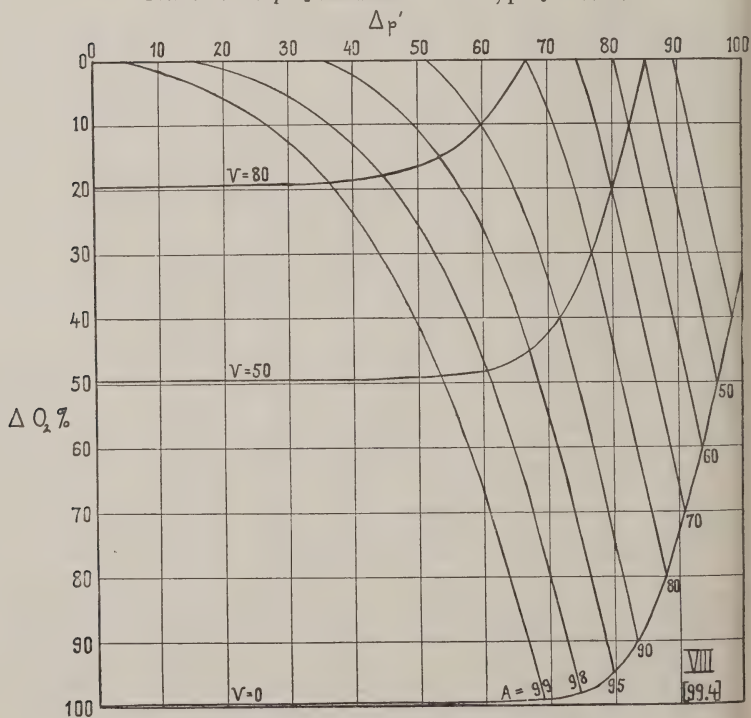


CHART VIII. pO_2 alveolar air = 110 mm.; $pH_s = 7.45$.

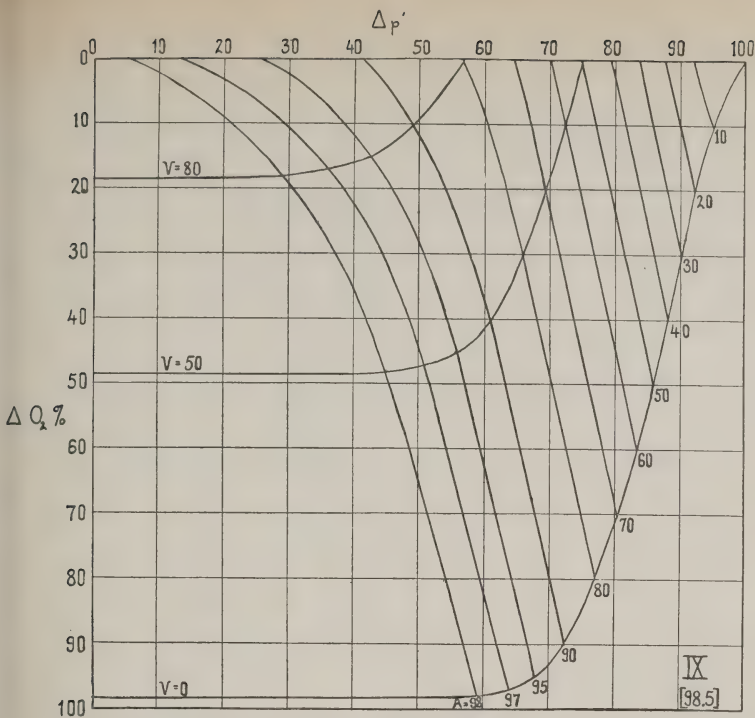


CHART IX. pO_2 alveolar air = 100 mm.; $pH_s = 7.45$.

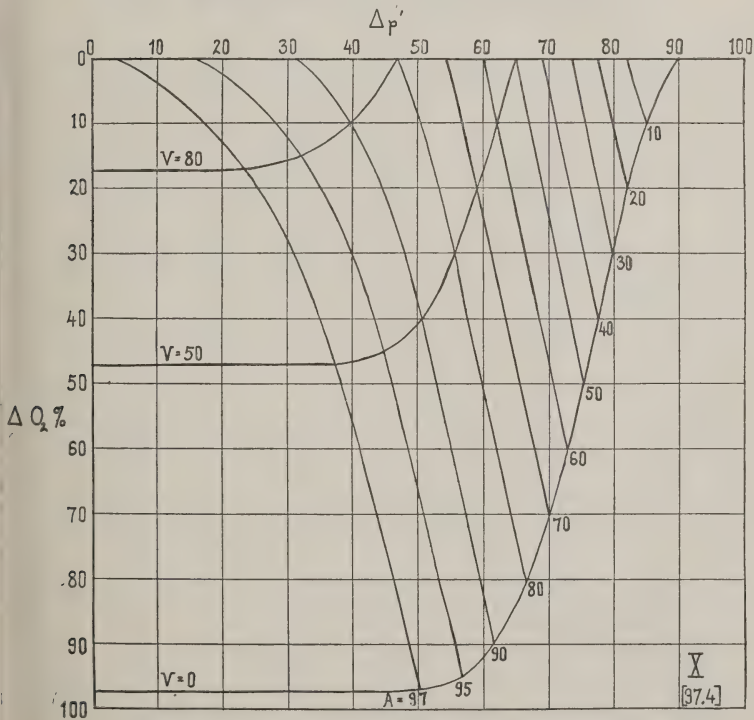


CHART X. pO_2 alveolar air = 90 mm.; $pH_s = 7.45$.

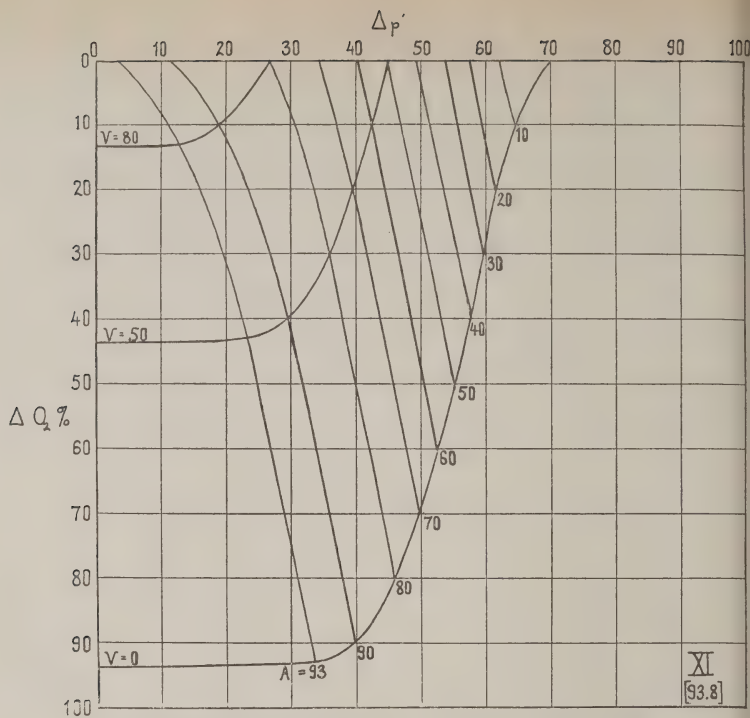


CHART XI. pO_2 alveolar air = 70 mm.; $pH_s = 7.45$.

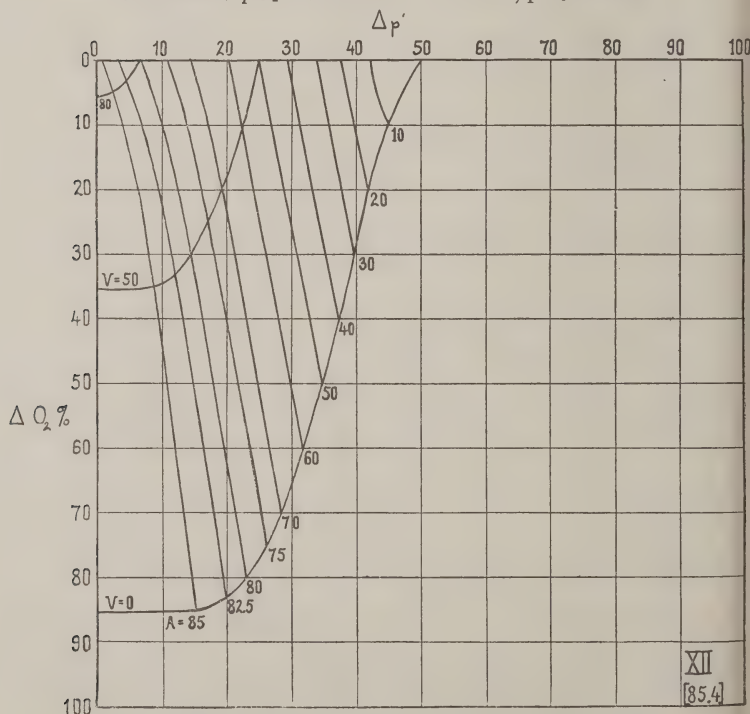


CHART XII. pO_2 alveolar air = 50 mm.; $pH_s = 7.45$.

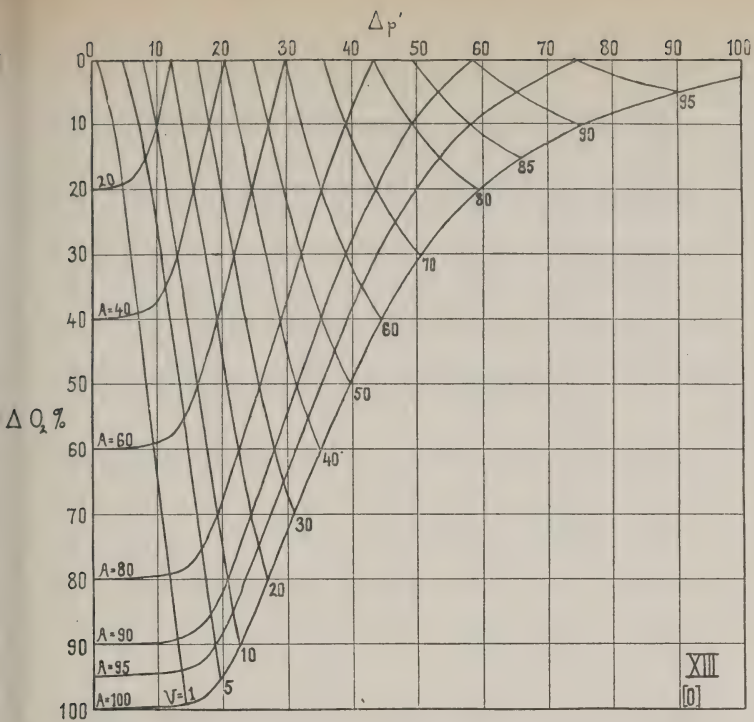


CHART XIII. pO_2 tissues = 0 mm.; $pH_s = 7.45$.

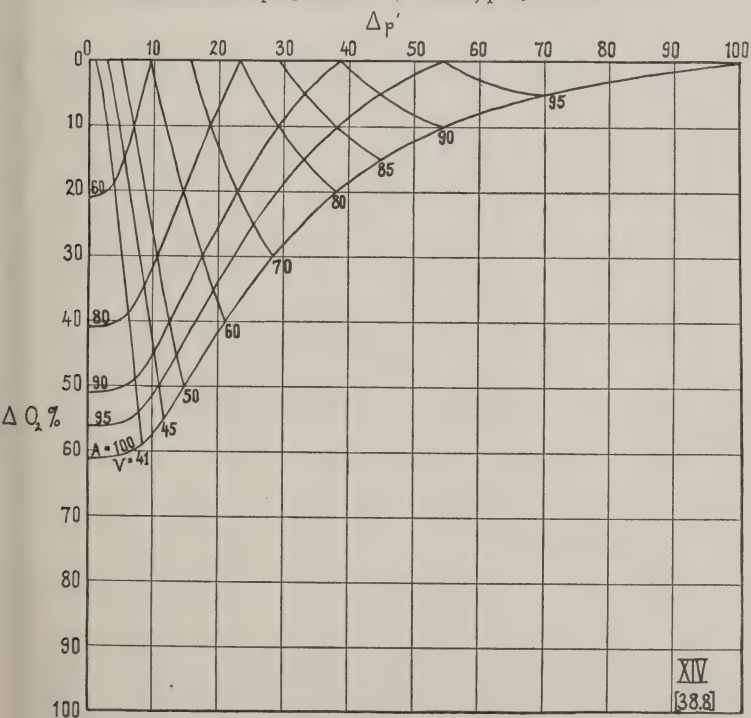


CHART XIV. pO_2 tissues = 20 mm.; $pH_s = 7.45$.

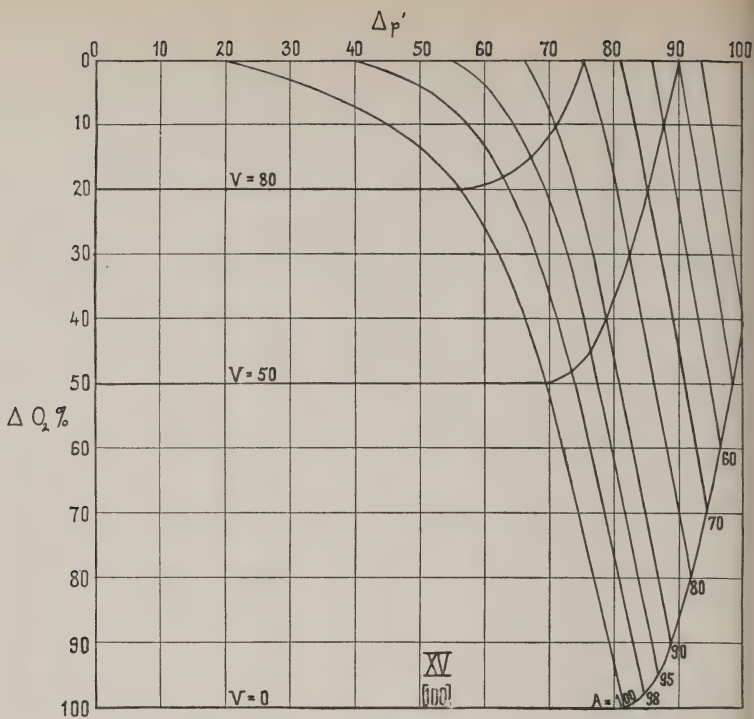


CHART XV. pO_2 alveolar air = 110 mm.; $pH_s = 7.65$.

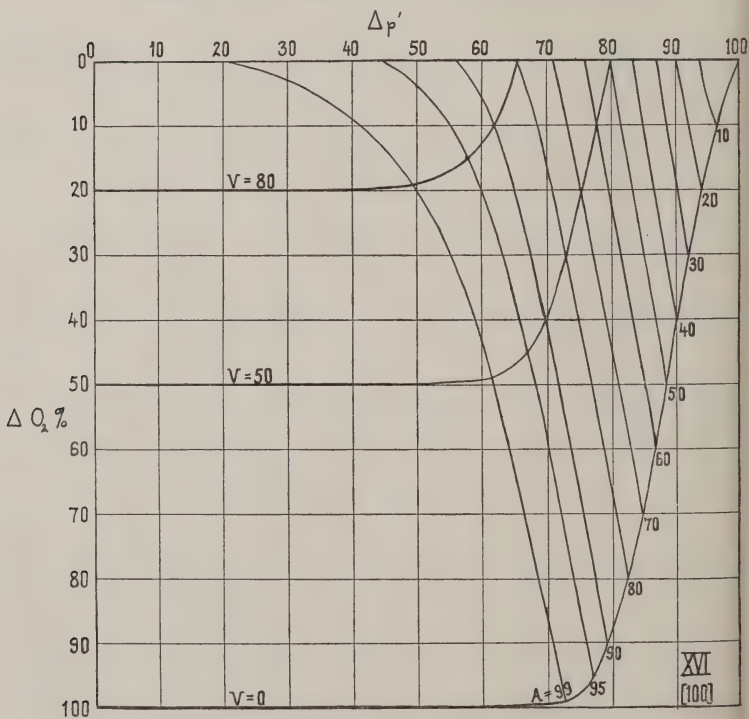


CHART XVI. pO_2 alveolar air = 100 mm.; $pH_s = 7.65$.

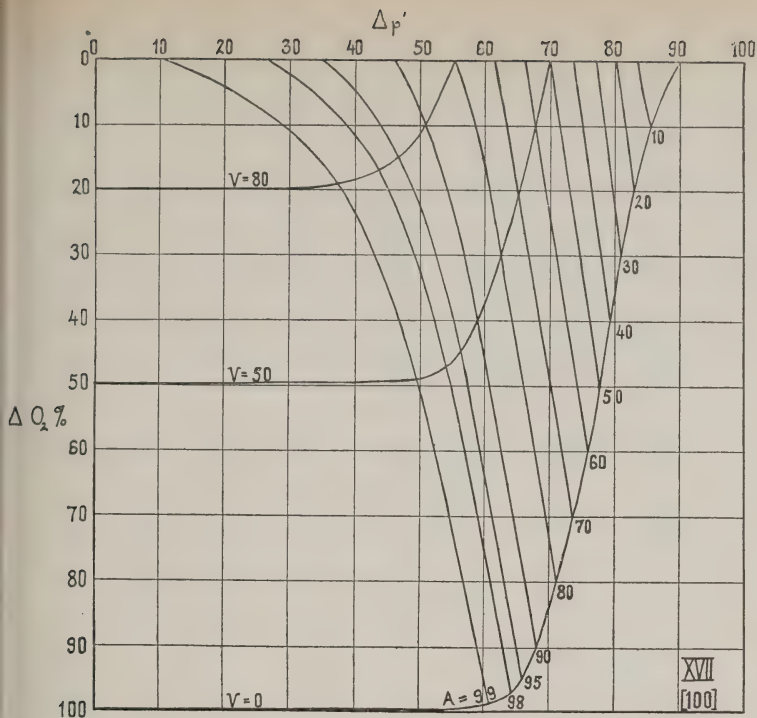


CHART XVII. pO_2 alveolar air = 90 mm.; pH_s = 7.65.

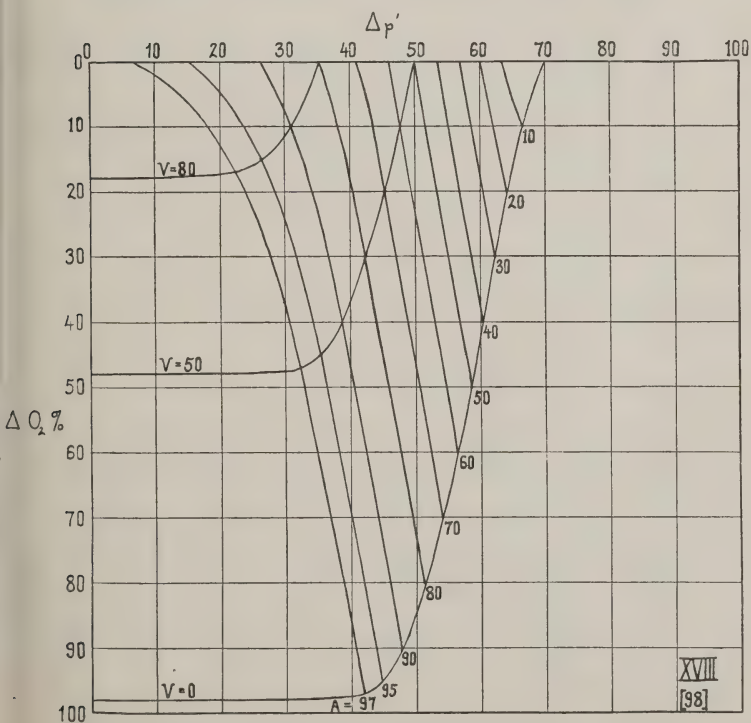


CHART XVIII. pO_2 alveolar air = 70 mm.; pH_s = 7.65.

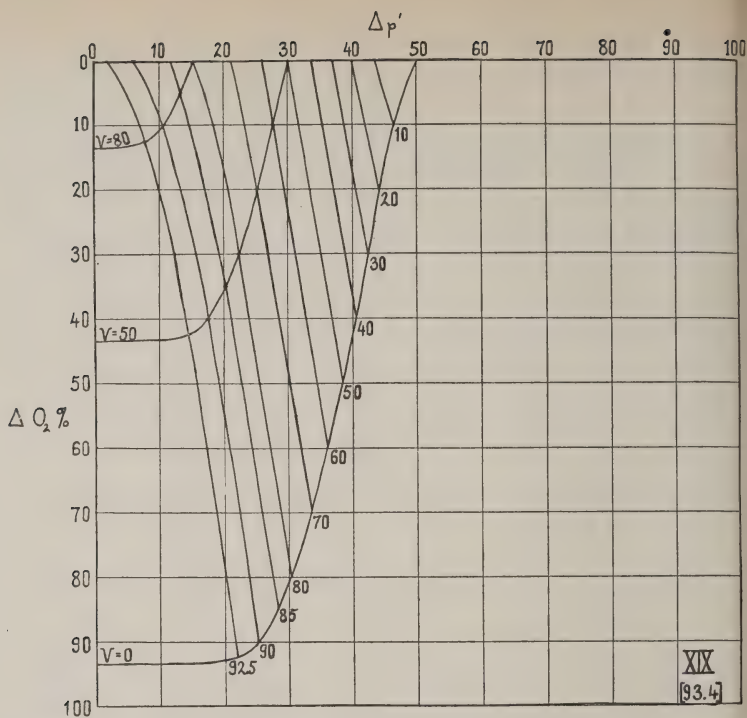


CHART XIX. pO_2 alveolar air = 50 mm.; $pH_s = 7.65$.

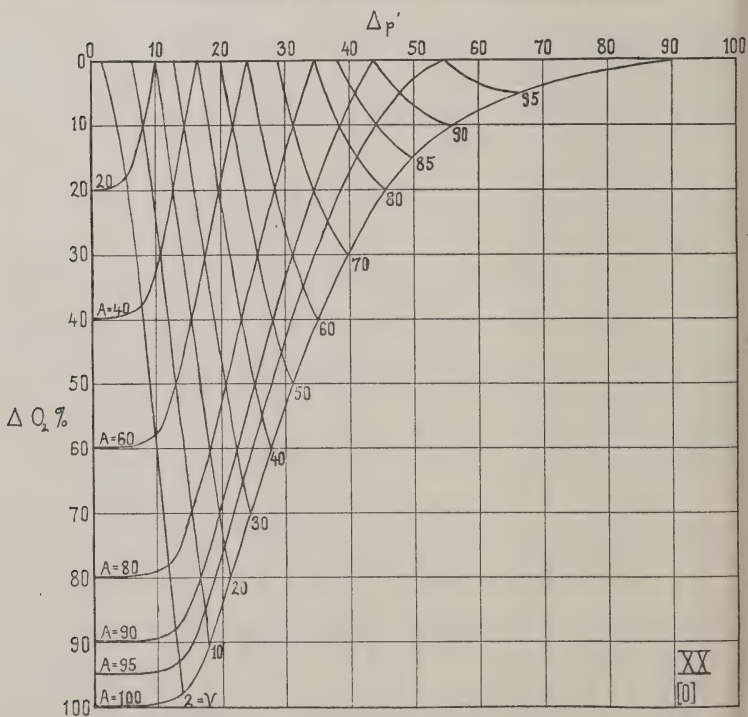


CHART XX. pO_2 tissues = 0 mm.; $pH_s = 7.65$.

Equations 1, 5, and 6, and the definition: $SHF = \frac{BF \times Hgb}{DC}$.

Equation 5 introduces a term, ΔO_2 cc., which is the difference in actual oxygen content (in cc. of O_2 contained per cc. of blood) between arterial and mixed venous bloods. The relation expressed is sufficiently obvious. Equation 6 is equally obvious. Throughout, it must be kept in mind that MR , DC , and BF re-

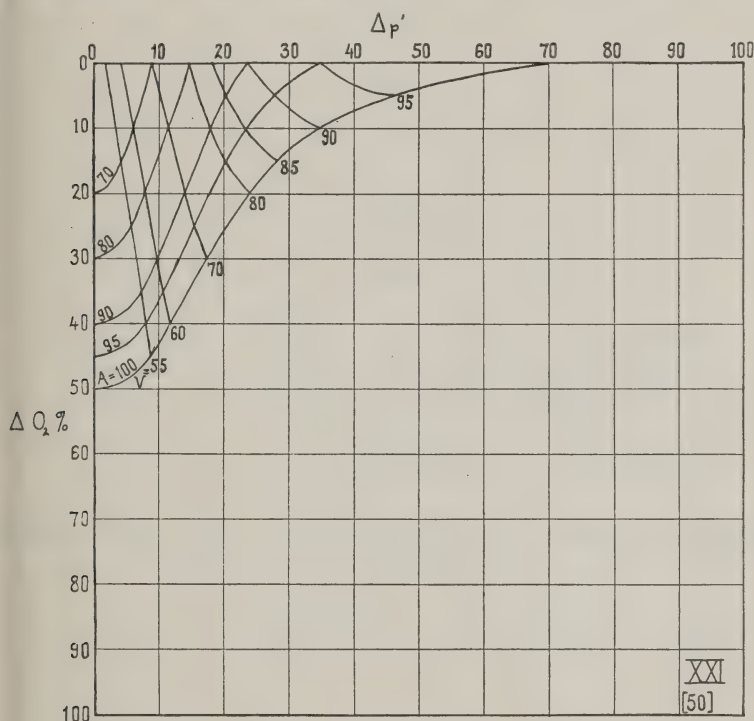


CHART XXI. pO_2 tissues = 20 mm.; $pH_s = 7.65$.

er to corresponding values for the specified anatomical region considered in any case, whether this be a single capillary, a single organ, or the lungs as a whole, or the tissues (other than lungs) as a whole. In the last two cases MR would represent the total oxygen consumption of the individual per minute, in short, the metabolic rate; and similarly BF would be the total blood flow per minute, and DC would be the total diffusing capacity of lungs

or tissues, according to which portion of the circulation was under consideration.

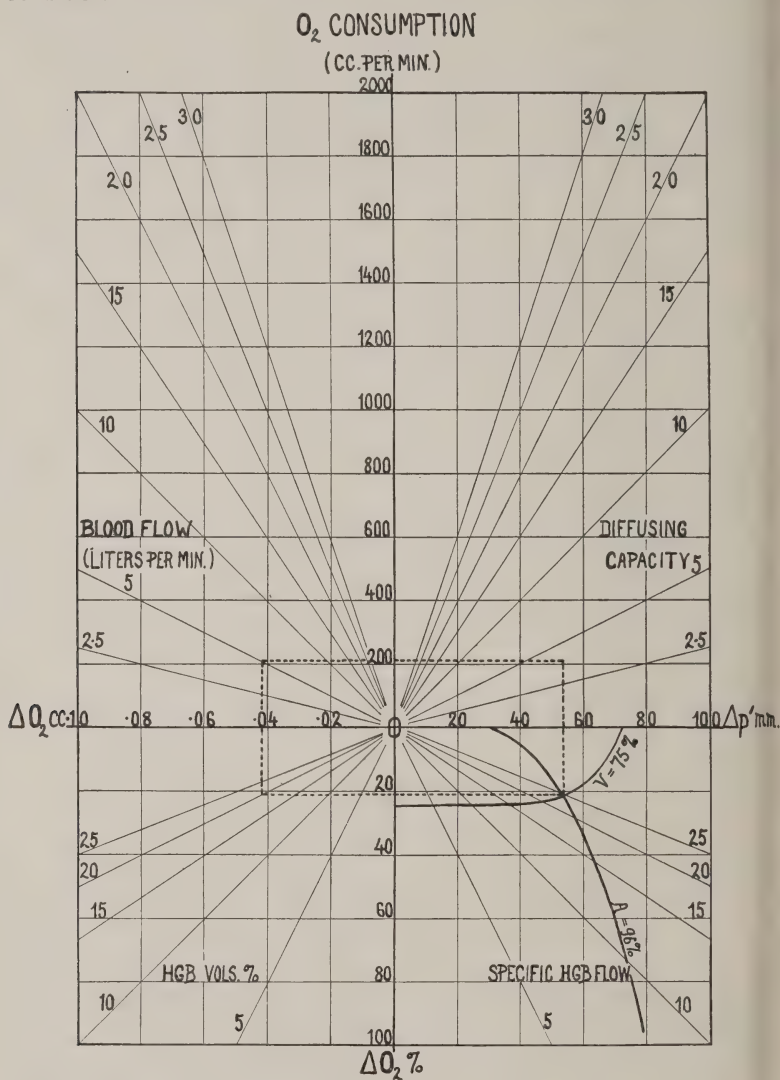


FIG. 1.

Now each of the equations just given can be expressed in simple graphic form, and the fact that some variables occur twice makes

it possible to arrange four adjoining graphs in such a way that there will be but two sets of ordinates (for MR and ΔO_2 per cent) and two sets of abscissæ (for $\Delta p'$ and ΔO_2 cc.). The arrangement will be seen in Fig. 1 of this paper—to be spoken of as the figure in contradistinction to the charts already described. It will be noticed that the lower right quadrant of the figure has the same coordinate background as that of the charts, and hence any one of these charts can be expanded into the form of Fig. 1, or a specific chart may be thought of as placed in the lower right quadrant of the figure. In this quadrant, the rectangular coordinates being ΔO_2 per cent and $\Delta p'$, SHF is represented by radiating coordinates as indicated by Equation 4. Lines for SHF could also have been drawn on the charts. The lower left quadrant will be seen to correspond with Equation 5, Hgb being represented by radiating lines. The upper left quadrant corresponds to Equation 6, with BF represented by the radiating lines. Lastly, the upper right quadrant corresponds to Equation 1, with DC represented by radiating lines.

On such a figure, which defines, in part at least, the physico-chemical background of the process of oxygen transport, the actual precise conditions prevailing may be delineated for any observed case. By way of recapitulation, the actual procedure for conditions in the lung may be briefly detailed, an example from the previous paper being chosen.

In the beginning it was mentioned that the function of oxygen transport would be described in terms of the operation of a system with six degrees of freedom. From an experimental point of view the first six variables to be mentioned may be selected as the independent ones, and the system defined as follows: First find

$$(1) pO_2 \text{ alveolar air} = 110 \text{ mm.}$$

$$(2) pH_s = 7.45$$

These two determinations indicate the proper chart to refer to—in this case Chart VIII.

$$(3) A \text{ per cent} = 96$$

$$(4) V \text{ per cent} = 75$$

These two findings now fix a point on Chart VIII, and consequently a point in the lower right quadrant of Fig. 1. From this

438 Oxygen Exchange, Blood, and Circulation

point on the figure two lines are drawn, one vertically upward, the other horizontally to the left. Now the determination of

$$(5) Hgb = 20 \text{ vols. per cent}$$

serves to fix, by the intersection of the radial line $Hgb = 20$ with the horizontal line just drawn, a point in the lower left quadrant. From this point another vertical line is drawn upward. Finally, the determination of the oxygen consumption

$$(6) MR = 210 \text{ cc. of } O_2 \text{ per minute}$$

completes the description of the system.

The following variables can now be obtained by simple calculation or graphical inspection.

$$(7) \Delta p' = 53 \text{ mm.}$$

$$(8) \Delta O_2 \text{ per cent} = 21 \text{ per cent}$$

$$(9) SHF = 25, \text{ or } SDC = 0.040$$

$$(10) \Delta O_2 \text{ cc.} = 0.042$$

$$(11) BF = 5 \text{ liters per minute}$$

$$(12) DC = 4.0$$

The dotted rectangle, which can be called the "lung rectangle," represents the above case as it would appear on Fig. 1. The figure should be labelled with the appropriate values for pO_2 alveolar air and for pH_s .

If it were possible to determine the oxygen pressure of the tissues, and if this pressure were uniform, the original experimental determinations mentioned above (with a value for pO_2 tissues substituted for pO_2 alveolar air) could be applied to the greater circulation. As it is, the best that can be done in practice is to assume some standard arbitrary value for pO_2 tissues. Thus relative values can be obtained for the other factors which might be of interest. For instance, to continue with the special case just discussed, assuming pO_2 tissues = 0, and neglecting for the moment the small difference in pH_s between arterial and venous bloods, one refers to Chart XIII. Then, utilizing values already stated above, one finds for the tissues:

$$(13) \Delta p' = 51 \text{ mm.}$$

$$(14) SHF = 24$$

$$(15) DC = 4.1$$

Other variables, represented by the left portion of the rectangle are identical for lungs and tissues in any given steady state. The only difference between the lung and tissue rectangles lies in the position of the line forming the right side of each rectangle.

If the oxygen pressure of the tissues were uniform, regardless of its actual value, the whole process of oxygen exchange and transport in lungs and tissues together could be described fairly accurately as the operation of a system with *seven* degrees of freedom. It is interesting to observe that when the conditions prevailing in the lung have been defined, all but one of the theoretically independent variables are approximately determined for the tissues as a whole.

Although forming no essential part of the present paper, it may be pointed out that the determination of the variables already mentioned leads to certain other factors which amplify the general description of the circulation. For example, if BF be multiplied by the systolic blood pressure, a good estimate of the effective mechanical work of the heart is obtained. Similarly, BF divided by the pulse rate yields a value for the volume output of each ventricle per beat. The blood flow, the blood pressure, and the viscosity of blood, taken together can lead to valuable information concerning the peripheral vessels and circulation.

DISCUSSION OF THE CHARTS WITH PARTICULAR REFERENCE TO THE ASSUMPTIONS INVOLVED.

It is now necessary to examine the conditions which must be fulfilled in order that application of the charts and the figure may be rigorous, and to examine the errors which are apt to occur as conditions depart from the ideal. The method of construction of the $\Delta p' - \Delta O_2$ per cent charts suffers from the limitations of any graphical procedure, but besides there may arise slight errors from three known sources.

1. Although it is supposed that the oxygen dissociation curve of hemoglobin is primarily determined by the pH and the temperature, other factors, such as the salts of the red blood corpuscle and the concentration of hemoglobin itself, may be involved, and especially in connection with the relation $pH_s - pH_c$ (7). Thus the pH_s values assigned to the charts are subject to future modi-

fication, or to the substitution of some other more significant variable such as pH_c , the activity of the hydrogen ion in the red blood corpuscle.

2. By expressing actual dissociation curves in the percentage saturation form (taking the total oxygen capacity to include the free dissolved oxygen, and calling this 100 per cent saturation), the fact that variations in the hemoglobin content alter the shape of the curve is neglected. There is thus an error, which in the extreme case assigns to pure serum an oxygen dissociation curve typical of ordinary whole blood instead of the simple straight line relation between $p\text{O}_2$ and dissolved O_2 . With blood poor in hemoglobin, the values of $p\text{O}_2$, at a given total content of oxygen, would actually be greater than the values of $p\text{O}_2$ used in the calculation of the charts. In anemia, then, $\Delta p'$ in the lungs would be actually less, and $\Delta p'$ in the tissues would be greater, than the corresponding values calculated from the charts; and conversely in polycythemia. To avoid this error much additional labor would be required and many more charts; fortunately, since the amount of dissolved oxygen is small in comparison with the combined oxygen in cases within the range of hemoglobin content compatible with life, the error from this source is insignificant.

3. In the integration of the amount of oxygen passing across the capillary wall, it is assumed that blood follows a path, or goes through changes, expressed by the oxygen dissociation curve, in other words that the oxygen content of the blood is determined by the *equilibrium* oxygen pressure of the blood (and *vice versa*), and moreover that this relation is sufficiently accurately expressed by the oxygen dissociation curve at constant $p\text{CO}_2$ of blood.³ Obviously $p\text{CO}_2$ varies during the passage of blood through a capillary, and so does pH_s ; but reference to Figs. 111 and 112 of Henderson's paper (6) will show that the theoretical path of the blood in the *lung* is very close to the oxygen dissociation curve at a constant pH_s or at a constant $p\text{CO}_2$ if the *arterial* values of these factors are taken. In the *tissues* the path followed is very close to the curve at the pH_s or $p\text{CO}_2$ of the *venous* blood. This presumptive fact, as Henderson showed, is due to the exchange of CO_2 being much more rapid than the exchange of O_2 . The values

³ This slight inconsistency has been avoided in the preceding paper.

of $\Delta p'$, as obtained from the charts, will be too high as a result of neglecting this factor, but only in exceptional cases will the error be greater than about 1 mm., and usually it will be much less. The three sources of error so far mentioned may lead to a combined error of about 5 per cent in the value of $\Delta p'$ as calculated.

We come now to more indefinite sources of error. If the reaction between dissolved oxygen and hemoglobin is slow, or if the diffusion of oxygen into the red corpuscle is slow, compared to the process of diffusion across the capillary wall—a consideration suggested by the recent work of Hartridge and Roughton (8)—the path of the blood in a capillary will not follow the oxygen dissociation curve. At any point along a capillary in the lung, pO_2 of blood will be higher than the oxygen content would lead us to suppose, hence $\Delta p'$, as calculated, will be too high. In the tissues, pO_2 of blood will be lower than we should suppose, and hence $\Delta p'$ will again, as calculated, be too high. For the present, until the possible error involved can be properly corrected, we can only assume that the magnitude of this effect will be relatively constant.

The last question to be raised in respect to the underlying assumptions in our calculations concerns the extension of results, based on the theoretical properties of blood in a given capillary, to cover the lung as a whole or the tissues as a whole. The problem of the tissues is beset with difficulties. In the first place the oxygen pressure of the tissues cannot be accurately determined. Secondly, the oxygen pressure in the tissues along a capillary is probably not uniform, but higher at the end which receives the arterial blood. Thirdly, the mixed venous blood, derived from various organs in which pO_2 may be very different, offers scant information in respect to the precise prevailing conditions. However, marked variations, characteristic of the tissue circulation as a whole or of a significant portion of it, will be reflected in the composition of the mixed venous blood. Thus comparative data may be obtained, and for this purpose it may be useful to select the arbitrary value $pO_2 = 0$ as a reference standard for the oxygen pressure of the tissues.

In contrast to the diversity of conditions presented by the rest of the body, the lung offers a condition of relative uniformity. The oxygen pressure of the alveolar air can be obtained with considerable accuracy, and this pressure is presumably uniform along

the length of a capillary. In the lung there is practically only one case which cannot be accurately described from data on the mixed arterial blood; namely, a condition of *partial* stagnation. After a quantity of blood has nearly reached equilibrium with alveolar air, it may travel through any additional length of capillary without further detectable change in composition. If a considerable quantity of such blood mixes with blood which is not in equilibrium with alveolar air, the mixed blood will not reveal the existence of the superfluous length of capillary through which the first portion has passed. The values of $\Delta p'$, as determined by the data on such mixed arterial blood, will be too high, and consequently the values of the diffusing capacity will be too low. The superfluous capillary area will not be discovered unless other signs or symptoms suggest its existence.

Many abnormalities of the lung may be examined from a functional standpoint by means of the charts which have been given. Hypothetical examples, for instance examples characterizing a short-circuited circulation or a case of obstructed bronchus, may be chosen, and the consequences of these conditions may be estimated. In every case it will be found that lack of uniformity involves a loss of efficiency: either there is a superabundance of capillaries, or an unduly large blood flow through ill ventilated regions of the lung, or both. The problem suggested here will not be further discussed in this paper.

SUMMARY.

A general physicochemical background, over which the process of oxygen transport and diffusion runs its course, has been described quantitatively in terms of the operation of a system with six independent degrees of freedom.

The process has been studied as a function of (1) the metabolic rate, (2) the oxygen capacity of the blood, (3) and (4) the percentage saturations of arterial and mixed venous bloods, (5) the hydrogen ion activity of the serum, and (6) the oxygen pressure of the alveolar air (or the tissues). Certain other variables might, however, be substituted in this list.

More particularly, the relations between the coefficient of utilization, the capillary area across which diffusion takes place, the arterial and venous percentage saturations, and the "mean"

head of oxygen pressure across the capillary wall have been given for a range of combinations of values for the hydrogen ion activity of the serum and the oxygen pressure of the alveolar air and of the tissues.

A new term, the specific hemoglobin flow, has been introduced to emphasize the important physiological adjustment of the product, blood flow times oxygen capacity, to capillary area. This derived factor is the reciprocal of the equally significant factor, specific diffusing capacity.

In that the discussion and the treatment of the various factors aim at giving a fairly complete description of the conditions for the diffusion and transport of oxygen in the body, it is hoped that this description may afford a basis for testing the physiological rôle of the diffusion process, and the distribution of physiological function among the various factors under all sorts of environmental and pathological states.

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444 Oxygen Exchange, Blood, and Circulation

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THE EFFECT OF VARIATION IN IONIC STRENGTH ON THE APPARENT FIRST AND SECOND DISSOCIATION CONSTANTS OF CARBONIC ACID.

BY A. BAIRD HASTINGS AND JULIUS SENDROY, JR.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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The biological and chemical importance of carbonic acid has stimulated much work upon its first dissociation constant with the immediate object of using this constant for the calculation of the hydrogen ion concentration of biological solutions. Fewer investigations have had as their object the determination of the second dissociation constant.

The mathematical treatment of the behavior of solutions of strong electrolytes by Milner (1) and by Debye and Hückel (2) has brought to the empirical results obtained by Lewis (3) and his collaborators, and Brönsted and La Mer (4) a theoretical explanation which seems adequate. Experimental work has indicated that the deviation in the behavior of strong electrolytes in moderately dilute solution from their behavior in infinitely dilute solution can be approximately expressed in the following manner.

if α = the activity

γ = " " coefficient, and

c = " molal concentration of an ion, then

$\alpha = \gamma c$. Further, that the activity coefficient is related to the ionic concentration of the solution by the empirical formula

$$-\log \gamma = \beta v^2 \sqrt{\mu} \text{ where}$$

β = an empirically determined constant which has a value of approximately 0.50

v = the valence and

μ = the ionic strength and is defined as $\frac{1}{2} \sum cv^2$.

The theoretical considerations of Debye and Hückel led to an expression, which under certain limiting conditions, corresponded well with the above empirical equation.

In view of these developments and the desirability of knowing the activity of bicarbonate and carbonate ions for subsequent studies, we have endeavored to determine the effect of varying ionic strength on the apparent dissociation constants of carbonic acid.

FIRST DISSOCIATION CONSTANT.^a

The first dissociation constant of carbonic acid has been accurately determined by numerous investigators. Of the determinations based on conductivity measurements those of Walker and Cormack (5) and of Kendall (6) are probably the most reliable. The results of these workers, recalculated by Warburg (7), give the value for K_1 as 3.11×10^{-7} at 18° ; $pK_1 = 6.507$. Kendall's value for pK_1 is 6.656 at 0° and 6.460 at 25° . This represents a change in pK_1 per degree of 0.0078. Assuming that this temperature coefficient is valid up to 38° , the pK_1 of carbonic acid would be 6.36 at body temperature. Hasselbalch (8) in 1916, determined pK_1' electrometrically in bicarbonate solutions ranging in concentration from 0.005 to 0.05 N. The method for measuring electrometric pH in CO_2 -containing solutions at that time contained certain errors, which Warburg has pointed out in recalculating Hasselbalch's values for pK_1' . These values are consistently about 0.09 lower than Warburg's and about the same amount lower than ours. The reason for the discrepancy apparently lies in the differences in technique employed in the measurement of pH values. The most accurate determinations of the first dissociation constant by electrometric measurement appear to be those of Warburg. These results we have recalculated, using the solubility coefficient for CO_2 found in this laboratory, and have included in Fig. 1 with our own results.

The mass law equation for the ionization of carbonic acid in terms of activity is

$$\frac{\alpha_{\text{H}^+} \times \alpha_{\text{HCO}_3'}}{\alpha_{\text{H}_2\text{CO}_3}} = K_1$$

In the logarithmic form this becomes

$$(1) \quad \log \alpha_{\text{H}^+} + \log \alpha_{\text{HCO}_3'} - \log \alpha_{\text{H}_2\text{CO}_3} = \log K_1$$

where α represents the activity of each individual component. For $\alpha_{\text{HCO}_3'}$ one may write $\gamma_1 [\text{HCO}_3']$ where γ_1 denotes the activ-

ity coefficient of the HCO_3' ion. Furthermore, in the presence of a relatively large amount of NaHCO_3 one may write $\gamma_1 [\text{NaHCO}_3]$ instead of $\gamma_1 [\text{HCO}_3']$, assuming for the moment, that NaHCO_3 behaves as a strong electrolyte. Since the activity of a gas is proportional to its tension, one may write

$$\alpha_{\text{H}_2\text{CO}_3} = \frac{p_{\text{CO}_2}}{760} \times \frac{a_{\text{CO}_2}}{0.0224}$$

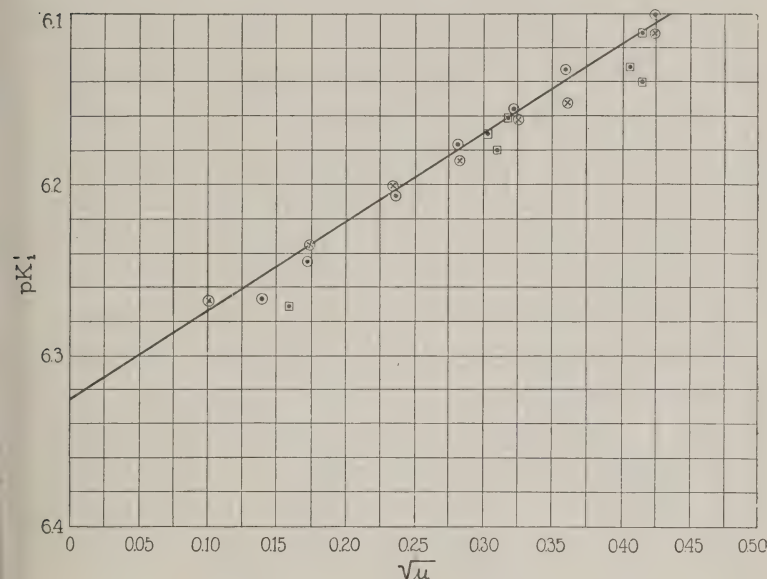


FIG. 1. Values of pK_1' are plotted as ordinates and of $\sqrt{\mu}$ as abscissæ. Points marked \circ and \otimes are from data given in the present paper. Those marked \square indicate data recalculated from Warburg's experiments. The line represents values of pK_1' calculated as $pK_1' = 6.33 - 0.5 \sqrt{\mu}$.

where p_{CO_2} denotes the tension of CO_2 with which the solution is in equilibrium and a_{CO_2} the solubility of CO_2 in the solution at that emperature. In conformity with the recent recommendations of Sørensen and Linderström-Lang (9) we shall write $p\alpha_{\text{H}}$ for $-\log \alpha_{\text{H}}$. With the above substitutions Equation 1 then becomes

$$\begin{aligned} 2) \quad -p\alpha_{\text{H}} + \log [\text{NaHCO}_3] - \log p_{\text{CO}_2} - \log \frac{a_{\text{CO}_2}}{760 \times 0.0224} \\ = \log K_1 - \log \gamma_1 \end{aligned}$$

$$3) \quad \text{We shall let } pK_1' = -\log K_1' = pK_1 + \log \gamma_1$$

Our experiments have been performed with the object of determining the relation between pK_1' (and consequently $\log \gamma_1$) and the ionic strength of the solution.

EXPERIMENTAL.

The experiments to be reported here represent the results of the determination of pK_1' in solutions of eight different ionic strengths. Each of these experiments was repeated with a freshly prepared solution.

The sixteen different solutions whose compositions are given in Table I were prepared from NaHCO_3 and NaCl of a high degree of purity. Each was rotated for 30 minutes in a water bath at 38° with hydrogen and CO_2 at a tension previously calculated to give the desired pH. The saturation was repeated for a second 30 minute period in order to insure equilibrium. The liquid phases were then separated by the technique described in a previous paper (10) and the following analyses were made.

Samples of the gas phase were transferred to the Haldane-Henderson gas apparatus and the tension of CO_2 was determined. The liquid phase, which was contained in filled glass bulbs over mercury, was analyzed for its CO_2 content in the Van Slyke (11) manometric gas apparatus. The $p\alpha_{\text{H}}$ was determined electrometrically at 38° in the Clark electrode vessel using the cell

Hg	HgCl	Saturated KCl	Saturated KCl bridge	Unknown solution	H_2	Pt
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The potential of the cell, with 0.1 N HCl in place of the unknown solution, was determined at the beginning of each day's experiment. This value of " ϵ_0 ", calculated on the assumption that the $p\alpha_{\text{H}}$ of 0.1 N HCl is 1.08, is given in each table. As pointed out by Cullen (12) this procedure serves as a daily calibration of the cell system used. The value 1.08, used as the $p\alpha_{\text{H}}$ of 0.1 N HCl, is taken from Lewis' value for the activity of H^+ in an HCl solution whose ionic strength is 0.1μ .¹ Values of $p\alpha_{\text{H}}$ are calculated from the equation

$$p \alpha_{\text{H}} = \frac{\text{E.M.F. (corrected to 1 atmosphere dry H}_2) - \epsilon_0}{0.06164}$$

¹Lewis and Randall (3), p. 332.

No.	p_{CO_2} mm.	Solubility coefficient of CO_2 .	H_2CO_3 mm. per l.	CO_2 mm. per l.	NaHCO_3 mm. per l.	$\frac{\log \text{NaHCO}_3}{\text{H}_2\text{CO}_3}$	pH	pK ₁ '	NaCl mm. per l.	μ	$\sqrt{\mu}$
1	61.6	0.552	1.997	12.25	10.25	0.710	6.977	6.267	0.00	0.01025	0.1012
2	65.6	0.552	2.127	12.40	10.27	0.684	6.950	6.266	0.00	0.01027	0.1013
3	117.6	0.550	3.80	23.97	20.17	0.725	6.990	6.265	0.00	0.02017	0.1418
4*	105.4	0.550	3.407	24.12	20.71	0.784	6.960	6.176	0.00	0.02071	0.1439
5	178.6	0.549	5.76	35.56	29.80	0.714	6.957	6.243	0.00	0.02980	0.1726
6	172.8	0.549	5.57	35.62	30.05	0.732	6.967	6.235	0.00	0.03005	0.1734
7	158.1	0.546	5.07	35.10	30.03	0.772	6.976	6.204	24.83	0.05486	0.2342
8	158.5	0.546	5.083	34.96	29.88	0.768	6.970	6.202	24.83	0.05471	0.2343
9	152.3	0.544	4.87	35.12	30.25	0.793	6.970	6.177	49.65	0.07990	0.2827
10	152.4	0.544	4.87	34.86	29.99	0.789	6.975	6.186	49.65	0.07964	0.2822
11	145.0	0.541	4.61	34.75	30.14	0.815	6.970	6.155	74.43	0.10457	0.3234
12	150.3	0.541	4.775	34.90	30.12	0.800	6.962	6.162	74.43	0.10455	0.3233
13	140.5	0.538	4.44	34.86	30.42	0.836	6.970	6.134	99.34	0.12976	0.3602
14	140.1	0.538	4.427	34.46	30.03	0.831	6.984	6.153	99.34	0.12937	0.3597
15	136.8	0.532	4.28	34.60	30.32	0.850	6.950	6.100	149.00	0.17932	0.4235
16	131.9	0.532	4.122	34.70	30.58	0.870	6.980	6.110	149.00	0.17958	0.4238

In odd numbered experiments $\epsilon_0 = 0.2364$; in even numbered experiments $\epsilon_0 = 0.2351$.

* Loss of CO_2 from gas phase. Not plotted in Fig. 1.

450 Dissociation Constants of Carbonic Acid

In Table I are given the results of our analyses. The H_2CO_3 is calculated from the CO_2 tension. This is subtracted from the total CO_2 leading to the values for NaHCO_3 .

In the ninth column are given the values of pK_1' calculated from these data by means of Equation 2. The results of our determinations, together with those found by Warburg, are given in Fig. 1, in which the values pK_1' are plotted as ordinates and the $\sqrt{\mu}$ as abscissæ.

It will be seen that most of the points lie on a straight line which intersects the ordinate at 6.33 and has a slope of 0.5. The equation of this line is therefore

$$\text{pK}_1' = 6.33 - 0.5 \sqrt{\mu}$$

Combining Equation 3 with this and substituting 6.33 for pK_1 we obtain

$$-\log \gamma_1 = 0.5 \sqrt{\mu}$$

This empirical equation apparently fits the experimental data even when the ionic strength of the solution is as great as 0.180. Such correspondence would not have been expected from the theory of Debye and Hückel because it is known that in solutions whose ionic concentration is greater than 0.10μ , correction should be made for the dimensions of the ions present and for the altered dielectric properties of the solvent. This would cause the theoretical line to bend toward the abscissa and our actual points lie slightly above such a curve. It is shown clearly, however, that the curve relating $-\log \gamma_1$ and $\sqrt{\mu}$ is linear and has a slope of the order of magnitude predicted by the Debye and Hückel theory in the region of moderately dilute solutions.

SECOND DISSOCIATION CONSTANT.

Determinations of the second dissociation constant of carbonic acid have been made by McCoy (13), Seyler and Lloyd (14), Bjerrum and Gjaldbaek (15), and others. McCoy's results, as recalculated by Stieglitz (16), led to a value of $K_2 = 5.4 \times 10^{-11}$ or $\text{pK}_2 = 10.27$ at 25° ; Seyler and Lloyd found $K_2 = 4.3 \times 10^{-11}$ or $\text{pK}_2 = 10.37$; and Bjerrum and Gjaldbaek give $K_2 = 6.0 \times 10^{-11}$ or $\text{pK}_2 = 10.22$ at 25° . Although it was recognized by McCoy that the concentration of the solution in which the con-

stant was determined affected the constant obtained, no systematic study of the effect of varying ionic strength on the second dissociation constant has been made.

As in the case of the first dissociation constant we are here concerned with the mass law equation,

$$(4) \quad \frac{\alpha_{H^+} \times \alpha_{CO_3''}}{\alpha_{HCO_3'}} = K_2$$

which in the logarithmic form becomes

$$(5) \quad \log \alpha_{H^+} + \log \alpha_{CO_3''} - \log \alpha_{HCO_3'} = \log K_2$$

Substituting $\gamma_2[CO_3'']$ and $\gamma_1[HCO_3']$ for $\alpha_{CO_3''}$ and $\alpha_{HCO_3'}$ respectively,

Equation 5 may be written

$$(6) \quad \log \alpha_{H^+} + \log \gamma_2 [CO_3''] - \log \gamma_1 [HCO_3'] = \log K_2$$

We have shown in the previous section that if $[HCO_3']$ be taken equal to $[NaHCO_3]$ then $-\log \gamma_1 = 0.5 \sqrt{\mu}$. By making the assumption that Na_2CO_3 also is a strong electrolyte, so that $CO_3'' = [Na_2CO_3]$, Equation 6 may be rewritten

$$(7) \quad \log \alpha_{H^+} + \log [Na_2CO_3] + \log \gamma_2 - \log [NaHCO_3] + 0.5 \sqrt{\mu} = \log K_2$$

Transposing $\log \gamma_2$ and $0.5\sqrt{\mu}$ to the right-hand side, dividing through by minus one and substituting $p\alpha_H$ for $-\log \alpha_H$, Equation 7 becomes

$$p\alpha_H - \log [Na_2CO_3] + \log [NaHCO_3] = -\log K_2 + \log \gamma_2 + 0.5 \sqrt{\mu} = pK_2'$$

Our experimental work has had for its purpose the determination of pK_2' in solutions of different ionic strengths. From these results we have been able to determine the relation between $\log \gamma_2$ and the ionic strength.

EXPERIMENTAL.

As in the case of the first dissociation constant, sixteen different solutions were prepared, representing eight different ionic strengths. The CO_2 of each solution was determined gasometrically and the total alkali ($NaOH + Na_2CO_3 + NaHCO_3$) titri-

TABLE II.
The pK_2' of Carbonic Acid at 38° in Salt Solutions of Varying Ionic Strength.

No.	NaOH, Na ₂ CO ₃ , and NaHCO ₃ .	NaCl	NaOH	Na ₂ CO ₃ + NaHCO ₃ .	Total CO ₂ 38 (Na ₂ CO ₃ + NaHCO ₃).	Na ₂ CO ₃	NaHCO ₃	log Na ₂ CO ₃ NaHCO ₃	$p\alpha_H$	pK_2'	μ	$\sqrt{\mu}$
	<i>m.-Eq. per l.</i>	<i>mm per l.</i>	<i>mm per l.</i>	<i>m.-Eq. per l.</i>	<i>mm per l.</i>	<i>mm per l.</i>	<i>mm per l.</i>					
1	15.35	0.00	0.40	14.95	10.07	4.88	5.19	-0.027	10.026	10.053	0.02023	0.1422
2	15.35	0.00	0.40	14.95	10.02	4.93	5.09	-0.014	10.027	10.041	0.02028	0.1424
3	30.10	0.00	0.39	29.71	19.76	9.95	9.81	0.006	10.005	9.999	0.04005	0.2001
4	30.10	0.00	0.40	29.70	19.78	9.92	9.86	0.003	10.010	10.007	0.04002	0.2000
5	45.13	0.00	0.37	44.76	29.59	15.17	14.42	0.022	9.969	9.947	0.06030	0.2455
6	45.20	0.00	0.38	44.82	29.66	15.16	14.50	0.019	9.979	9.960	0.06036	0.2457
7	45.20	24.83	0.34	44.86	29.56	15.30	14.26	0.031	9.914	9.883	0.08535	0.2922
8	45.20	24.83	0.35	44.85	29.57	15.28	14.29	0.030	9.932	9.902	0.08532	0.2921
9	45.27	49.65	0.31	44.96	29.65	15.31	14.34	0.029	9.867	9.838	0.11025	0.3320
10	45.20	49.65	0.31	44.89	29.47	15.42	14.05	0.041	9.872	9.831	0.11029	0.3321
11	45.13	74.43	0.29	44.84	29.80	15.04	14.76	0.008	9.836	9.828	0.13462	0.3669
12	45.27	74.43	0.30	44.97	29.54	15.43	14.11	0.039	9.846	9.807	0.13515	0.3676
13	45.20	99.34	0.27	44.93	29.67	15.26	14.41	0.025	9.797	9.772	0.15983	0.3998
14	45.20	99.34	0.27	44.93	29.45	15.48	13.97	0.045	9.797	9.752	0.16005	0.4001
15	45.27	149.00	0.25	45.02	29.64	15.38	14.26	0.033	9.753	9.720	0.20968	0.4579
16	45.20	149.00	0.25	44.95	29.58	15.37	14.21	0.034	9.752	9.718	0.20960	0.4578

metrically. Using Michaelis' (17) value of 13.475 for pK_w at 38°, $p\alpha_{OH}'$ was obtained. From the activity coefficient of OH' in solutions of different ionic strength, as given in Lewis and Randall, the $NaOH$ concentration was calculated.

From the ratio of $Na_2CO_3 + NaHCO_3$ to total CO_2 the Na_2CO_3 and $NaHCO_3$ were calculated. A ratio of Na_2CO_3 to $NaHCO_3$ was chosen which gave the maximum accuracy and was sufficiently high so that the H_2CO_3 concentration could be neglected. The

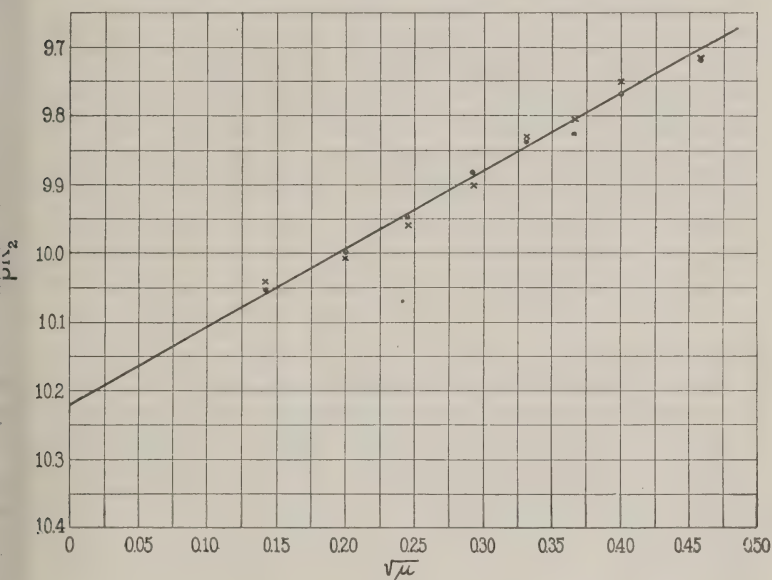


FIG. 2. Values of pK_2' are plotted as ordinates and of $\sqrt{\mu}$ as abscissæ. The line represents values of pK_2' calculated as $pK_2' = 10.22 - 1.1\sqrt{\mu}$. The points represent experimental results.

$p\alpha_H$ of each solution was determined electrometrically using as the values found with 0.1 N HCl at the beginning of each experiment.

From these data and Equation 8, we have calculated pK_2' for each solution. Our results are tabulated in Table II and graphically represented in Fig. 2 with pK_2' as ordinates and $\sqrt{\mu}$ as abscissæ. The equation which satisfies this line is

$$pK_2' = 10.22 - 1.1 \sqrt{\mu}.$$

454 Dissociation Constants of Carbonic Acid

Combining with this Equation 8, and putting $\text{pK}_2 = 10.22$, we obtain

$$-\log \gamma_2 = 1.6 \sqrt{\mu}$$

If the simple relationship $-\log \gamma = 0.5 \nu^2 \sqrt{\mu}$ held for the carbonate ion one might expect the relation between $-\log \gamma_2$ and the $\sqrt{\mu}$ to be

$$-\log \gamma_2 = 0.5 \times 2^2 \sqrt{\mu} = 2 \sqrt{\mu}$$

It is seen that the slope over the range of concentrations with which we worked is somewhat less than that expected from the theory. In view of the fact that no account has been taken of the dimensions of the ions, or of the change in dielectric constant of the solution at the higher concentrations, it is felt that the correspondence of our results with what might have been anticipated from theoretical considerations is satisfactory. As in the case of γ_1 , the linear relationship between $-\log \gamma_2$ and $\sqrt{\mu}$ appears to hold to a concentration of $\mu = 0.16$.

CONCLUSIONS.

The first and second dissociation constants of carbonic acid have been determined at 38° in solutions of varying ionic strength.

When extrapolated to $\mu = 0.0$, the first dissociation constant was found to be 4.68×10^{-7} or $\text{pK}_1 = 6.33$. The activity coefficient of the bicarbonate ion, γ_1 , was found to be related to the ionic strength of the solution from $\mu = 0.01$ to $\mu = 0.18$ according to the equation

$$-\log \gamma_1 = 0.5 \sqrt{\mu}$$

The apparent first dissociation constant is related to the ionic strength of the solution according to the equation

$$\text{pK}_1' = 6.33 - 0.5 \sqrt{\mu}$$

The second dissociation constant of carbonic acid was found to be 6.03×10^{-11} or $\text{pK}_2 = 10.22$ at 38° . The activity coefficient of the carbonate ion, γ_2 , was found to be related to the ionic strength of the solution from $\mu = 0.02$ to $\mu = 0.16$ according to the equation

$$-\log \gamma_2 = 1.6 \sqrt{\mu}$$

The apparent second dissociation constant is related to the ionic strength of the solution according to the equation

$$pK_2' = 10.22 - 1.1 \sqrt{\mu}$$

These results are in harmony with the theory of the behavior of strong electrolytes in dilute solutions as elaborated by G. N. Lewis, Brönsted, and Debye and Hückel.

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VITAMIN B IN THE EXCRETA OF RATS ON A DIET LOW IN THIS FACTOR.*

By W. D. SALMON.

From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn.)

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In 1922-23 while studying the effect of the seed of the velvet bean on the growth of rats that were receiving a diet deficient in vitamin B,¹ the author observed certain irregularities in the results. The decline of control animals on the basal diet and the response to the addition of varying percentages of beans were not always as uniform as they should have been. At that time the animals were kept on fine mesh screens which prevented access to the bedding but retained the feces. The cages were thoroughly cleaned and sprayed with disinfectant each week. Under these conditions the bottoms of some of the cages were kept rather moist and a slight growth of mould was apparent on the feces at times. As a sanitary measure the screens were replaced by large mesh screens. These were raised 2 inches from the bottoms of the pans under the cages. This allowed the feces to drop through, out of reach of the rats. A check in the rate of growth of several groups of rats was observed. This was particularly noticeable among the rats that were receiving diets low in vitamin B.

About that time a paper by Steenbock, Sell, and Nelson (1) showed that the consumption of excreta by rats in vitamin B determinations involved considerable error. They reported that the diet must contain twice as much vitamin B when the rats are

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¹ The velvet bean studies mentioned were made in cooperation with Dr. Emerson R. Miller, Research Chemist of this station. The results will appear soon. Dr. Miller also furnished the extracted beans for the tests reported in this paper. The author expresses his appreciation.

kept on screens and that about 60 per cent of the ration must be grain to furnish sufficient vitamin B for maximum growth of the rat.

The necessity for keeping animals on screens has been questioned in a recent paper by McCollum, Simmonds, and Becker (2). It seems doubtful whether their conclusions are justified by their findings. "The animals on the screen on the vitamin B-deficient diet were in much poorer condition than were those kept on shavings." It was further shown that more dry matter in feces was collected from the rats kept on shavings. It is possible that this was due to the supplemental effect of the feces which resulted in a larger intake of feed.

That the feces of rats may be rich in vitamin B even when the diet consumed is low in this factor is shown by the following data.

EXPERIMENTAL.

The basal diet used in these experiments was casein 18, salt No. 185 3.7, agar 2, rice powder 71.3, butter fat 5 (3). This was fed for a preliminary period of 3 weeks. The substance to be tested for vitamin B was then added, replacing an equal amount of rice powder. The method of caging varied in the different experiments.

Fig. 1 shows that fecal consumption may introduce a source of significant error under certain conditions. In this experiment the rats were caged individually on fine mesh screens which retained the feces. The cages were cleaned weekly. All the rats were losing weight on the basal diet despite the fact that feces were available. At the end of 3 weeks the diet was supplemented with 20 per cent of velvet beans. One group of rats received raw velvet beans while the other group received velvet beans that had been extracted in a Soxhlet apparatus with 95 per cent alcohol for 24 hours. There was an immediate response in both groups the rats receiving the extracted beans growing almost as rapidly as those receiving the raw beans. At point X the screens were replaced by large mesh screens which prevented access to the excreta. The rats receiving the raw beans continued to grow but at a slower rate. The rats receiving the extracted beans declined rapidly after the screens were changed. That vitamin B

as the lacking factor is shown by the resumption of growth in period D when two rats received yeast in addition to the extracted bean diet.

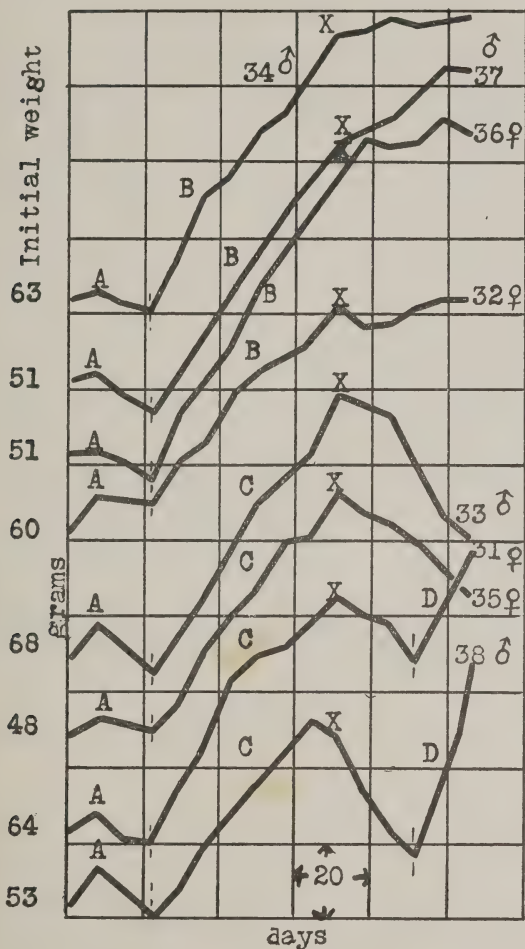


FIG. 1. Fecal consumption may introduce significant error in determinations of vitamin B.

A = basal diet only; B, basal diet with 20 per cent raw velvet beans; C, basal diet with 20 per cent velvet beans extracted in a Soxhlet apparatus 2 hours with 95 per cent alcohol; D, same as C with 5 per cent yeast added; X, screens changed, excreta not available.

Fig. 2 shows that the above diet (basal with 20 per cent of extracted velvet beans) was not capable of supporting growth when the excreta were not available. In this experiment the rats were caged on a raised screen (2 meshes to the inch). Suspended under this and out of reach of the rats was a fine mesh screen which retained the feces which were collected for the third experiment. The decline of the rats in the second experiment was comparable with the decline on the basal diet and the rats were in very poor condition after 5 weeks on the experimental diet. (The 3 week preliminary period on the basal diet was omitted in this experiment.) Each rat then received 0.4 gm of dried yeast daily for 2 weeks and made marked growth. This was followed by another period (7 weeks) on the extracted bean diet.

In the second experiment the feces were discarded for 10 days. After this they were collected each morning and dried in the air oven for 6 hours at 120°C. The feces were again discarded during and 1 week following the yeast period. During the last 6 weeks they were collected daily as before and dried in the air oven for 5 hours at 55°C. A portion of the dried excreta from each of the two periods was then moistened and allowed to stand in the laboratory for 1 week. A noticeable growth of mould appeared in this time. The moistened samples were again dried in the air oven at 55°C. This gave four samples of excreta which were tested in the third experiment.

1. Excreta dried 6 hours at 120°C.
2. Excreta dried 6 hours at 120°C., then kept moist 1 week, and redried at 55°C.
3. Excreta dried 5 hours at 55°C.
4. Excreta dried 5 hours at 55°C., then kept moist 1 week, and redried at 55°C.

Fig. 3 shows the effect of adding the dried excreta to the diet of rats that were declining on the basal diet. The feces were added at a concentration of 10 per cent and there was a marked response, the eight rats making an average weekly gain of 14 gm per rat for 3 weeks. The previous heating of the excreta for 6 hours at 120°C. apparently did not decrease the potency of the vitamin nor was there an increase due to the growth of mould.

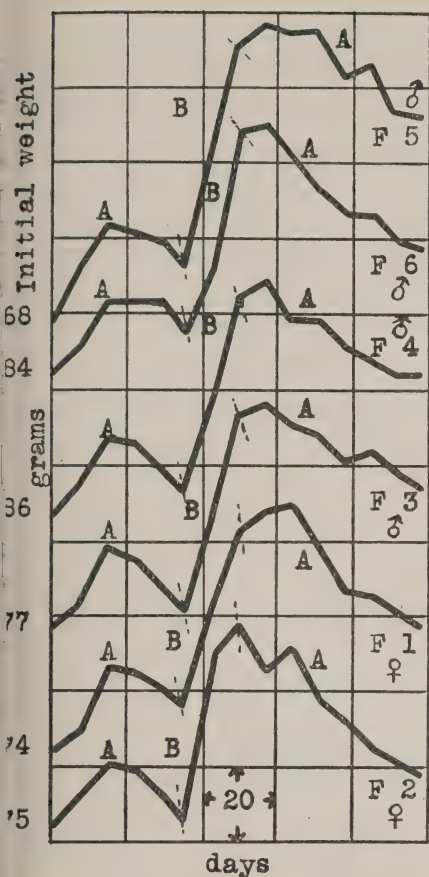


FIG. 2.

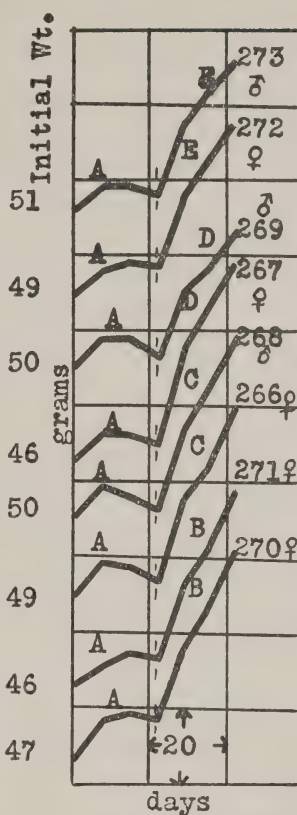


FIG. 3.

FIG. 2. Failure of growth on the basal diet with 20 per cent extracted velvet beans when the excreta were not available.

A = basal diet with 20 per cent velvet beans extracted in a Soxhlet apparatus 24 hours with 95 per cent alcohol; B, same as A with 0.4 gm. of dried yeast per rat daily.

FIG. 3. Excreta from rats on diet low in vitamin B as sole source of this factor.

A = basal diet only; B, 10 per cent excreta dried 6 hours at 120°C.; C, 10 per cent excreta dried 5 hours at 55°C.; D, 10 per cent excreta as in C, kept moist 1 week, then dried at 55°C.; E, 10 per cent excreta as in B, kept moist 1 week, then dried at 55°C.

DISCUSSION.

The necessity for keeping rats on screens in experiments involving vitamin B may become more apparent when natural foods are added to the diet than when the diet consists only of purified materials. Rats that were declining on the basal diet although they had access to the excreta, resumed growth when the diet was supplemented with 20 per cent of extracted velvet bean. When the feces were not available the decline was comparable to that on the basal diet. This indicates that the extracted bean did not contain a demonstrable amount of vitamin B but that the increased the vitamin B content of the feces.

The growth of rats receiving 10 per cent of dried feces from the above diet was comparable with the growth of Steenbock's rats that received 40 per cent of corn or oats as the source of vitamin B (1).

The growth of moulds on the feces did not increase their vitamin content under the conditions of these experiments. The possibility of synthesis of the vitamin by intestinal bacteria remains. The addition of the extracted velvet beans to the basal diet may have increased the undigested food residues in the intestinal tract and thus made conditions more favorable for bacterial activities.

Apparently, efforts should be made to reduce fecal consumption to a minimum in determinations of vitamin B. It is probably impossible to prevent it entirely. Even when rats receiving vitamin B-deficient diet are caged individually on screens, they will catch their own feces and eagerly devour them.

SUMMARY.

1. Rats on a diet deficient in vitamin B made marked growth when they had access to their excreta.
2. The excreta contained more vitamin B than corn or oats.
3. The consumption of feces by rats on diets low in vitamin B may result in significant error.

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SYNTHETIC NUCLEOSIDES.

I. THEOPHYLLINE PENTOSIDES.

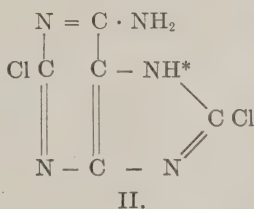
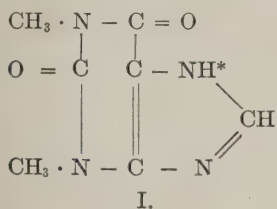
BY P. A. LEVENE AND HARRY SOBOTKA.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

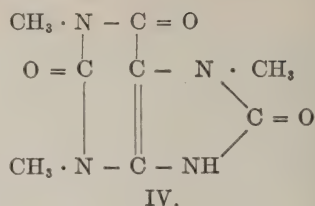
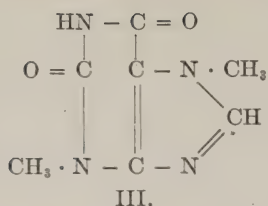
(Received for publication, June 30, 1925.)

Our knowledge of the details of the structure of purine nucleosides is based principally on analytical data. A recent publication by P. A. Levene (1) in which the methylation of xanthosine by means of diazomethane was reported gave additional data. All the analytical figures seem to indicate that in purine nucleosides the sugar is attached to atom (7) of the purine.

Evidence in favor of this view may be found in the synthetic work of E. Fischer and B. Helferich (2, 3, 4). These workers, immediately after the discovery of the nucleosides by Levene and Jacobs, undertook the synthesis of these substances. A review of the entire series of substances prepared by Fischer and his co-workers brings out the fact that they may be classified in two groups. Substances of one group resemble natural nucleosides, whereas others are distinctly different.



In the case of 1,3-dimethyl-2,6-dioxy purine (theophylline) (Formula I) and of 2,8-dichloroadenine (II) only the hydrogen in position (7), marked by an asterisk(*), can be replaced; position (9), though possible, is less probable. Hence, there can be no doubt as to the place of substitution. On the other hand, in theobromine (III) and in hydroxycaffeine (IV):



the free NH group (positions (1) and (9) respectively) is linked to one carbonyl in position (8) (in IV) or to two carbonyls in positions (2) and (6) (in III). Thus, during the formation of the silver salt enolization may take place, in which case the condensation leads to substitution in one of the C-O- groups.

The glucosides of theobromine and hydroxycaffeine belong to the second class of substances. They are extremely unstable even towards boiling water. The tetracetylglucoside of hydroxycaffeine was found to be so unstable that it could not be deacetylated at all without opening the sugar purine linkage. Thus these substances behaved unlike the natural nucleosides.

The synthetic glucosides of theophylline and dichloroadenine belong to the other class. The latter was used for the preparation of adenine, hypoxanthine, and guanine glucosides. The substances of this group showed a marked resistance both towards acid hydrolysis and towards alkaline hydrolysis. In this respect these substances resembled the naturally occurring nucleosides. However, this work of Fischer and his coworkers dealt principally with hexose nucleosides and only in a minor degree with pentose nucleosides. And yet, for the present, more importance is attached to the latter, inasmuch as for the present nucleosides have been isolated only from pentose nucleic acid. It seemed particularly expedient to prepare the pentosides of theophylline. in view of the fact that, as already mentioned, the natural guanosine had been converted into dimethyl xanthosine.

The xyloside and riboside of theophylline have now been prepared synthetically by us and the properties of the latter have been compared with those of the dimethyl xanthosine. The identity of both substances was proved by the uniformity of their optical rotation and by the agreement of the rate with which they underwent acid hydrolysis.

EXPERIMENTAL PART.

Acetobromoxylose (5).—Xylose tetracetate was dissolved in two to three times its weight of a commercial solution of hydrobromic acid in glacial acetic acid. After standing $1\frac{1}{2}$ hours at room temperature 3 parts of chloroform were added, and the mixture was washed with ice water until neutral to Congo red. The chloroform solution was dried over CaCl_2 sticks and was then concentrated under reduced pressure. The viscous residue was taken up in a small amount of ether. After standing a few hours at 0°C ., large transparent needles of the acetobromoxylose were collected. The yield was 65 per cent of the starting material or 10 per cent of the theory.

Acetobromoribose.—This substance had not as yet been prepared. It has less favorable properties than the analogous xylose derivative. When working with small quantities, it is advantageous to use 5 parts of HBr solution and 1 part of tetracetate. The substance should be washed not more than twice, then the dried chloroform solution is concentrated under reduced pressure, care being taken that the distillation is interrupted as soon as a slight darkening begins. An excess of absolute petroleic ether (b.p. $40\text{--}60^\circ$) is added to the concentrated solution. Due to its hygroscopic character, the product, which crystallizes in the cold, cannot be filtered on a Büchner funnel but must be centrifugalized and the centrifuge bottles placed at once in a vacuum desiccator over phosphorus pentoxide, soda-lime, and solid paraffin. Yield 50 to 55 per cent of the theory.

0.1814 gm. substance required 5.30 cc. 0.1 N AgNO_3 : 0.04235 gm. Br.

$\text{C}_{11}\text{H}_{18}\text{O}_7$ Br (339.04). Calculated. Br 23.57.

Found. " 23.35.

Triacetyl Theophylline Xyloside.—According to the directions given by E. Fischer and B. Helferich, 17 gm. of powdered theophylline silver were suspended in a solution of the calculated amount (19 gm.) of acetobromo-*d*-xylose in 150 cc. of xylene and boiled under a reflux condenser until a sample of the liquid gave no more reaction for bromine. The solution was filtered into another 150 cc. of cold xylene. By adding an excess of petroleic ether a white flocculent precipitate of triacetyl theophylline xylo-

side settled out. The compound was found to be very soluble in water, alcohol, ether, and toluene, but less soluble in ethyl acetate. The yield 16 gm. = 65 per cent of the theory. The rotation in methyl alcohol was

$$[\alpha]_D^{25} = \frac{-0.93^\circ \times 100}{1 \times 4.25} = -21.9^\circ$$

0.0983 gm. substance: 0.1766 gm. CO₂ and 0.0470 gm. H₂O.

C₁₈H₂₂O₉N₄ (438.21). Calculated. C 49.29, H 5.07.

Found. " 48.99, " 5.35.

Theophylline Xyloside.—10 gm. of the acetyl compound were dissolved in absolute methyl alcohol and a current of ammonia was passed through the thoroughly cooled solution. Overnight a

TABLE I.

Hydrolysis of a 2.45 Per Cent Solution of Theophylline Xyloside in 0.1 N HCl at 100° C.

Time.	α_D ($l = 2$ dm.)	Hydrolysis.	$k \times 10^4$
<i>min.</i>		<i>per cent</i>	
0	-1.42	0	
200	-0.98	23.5	5.82
350	-0.66	40.6	6.47
500	-0.44	52.4*	6.44
∞	+0.45	100	

* 0.1 cc. of the solution at this point gave with the Shaffer-Hartman method (6) 0.75 mg., calculated for glucose corresponding to 0.625 mg. of pentose. At 100 per cent hydrolysis 1.178 per cent of xylose would be released so this value of 53 per cent of sugar is in good accord with the optical determination.

hard white crust was formed, which gave after repeated recrystallization from methyl alcohol and water more than 4 gm. (60 per cent of the theoretical amount) of felt-like needles which melted at 229° (corrected). The substance analyzed as follows:

0.1013 gm. substance: 0.1704 gm. CO₂ and 0.0472 gm. H₂O.

0.0934 " " required (Kjeldahl) 12.10 cc. 0.1 N acid.

C₁₂H₁₆O₆N₄ (312.16). Calculated. C 46.14, H 5.16, N 17.95.

Found. " 45.88, " 5.21, " 18.13.

The optical rotation which was

$$[\alpha]_D^{25} = \frac{-0.57^\circ \times 100}{2 \times 1.00} = -28.5^\circ \text{ (in methyl alcohol)}$$

$$[\alpha]_{\text{D}}^{25} = \frac{-0.99^{\circ} \times 100}{1 \times 3.61} = -27.4^{\circ} \text{ (in water)}$$

remained unchanged in 0.5 N acid solution. In 0.5 N alkali the rotation became

$$[\alpha]_{\text{D}}^{25} = \frac{-1.48^{\circ} \times 100}{2 \times 1.805} = -41.0^{\circ}$$

The rate of hydrolysis is seen from Table I.

Triacetyl Theophylline Riboside.—5 gm. of freshly prepared acetobromoribose and 5 gm. of theophylline silver in 50 cc. of xylene were treated as above. The yield was 3.5 gm. The analysis showed that considerable amounts of the solvent were retained.

$$[\alpha]_{\text{D}}^{25} = \frac{-0.66^{\circ} \times 100}{2 \times 7.76} = -4.25^{\circ} \text{ (in methyl alcohol)}$$

Theophylline Riboside.—The crude triacetate was deacetylated by means of NH_3 in methyl alcohol. Like the other ribose derivatives, the theophylline riboside differs from the corresponding derivatives of other pentoses, particularly from those of xylose, by its extremely high solubility and hygroscopic character. Since also with prolonged standing no precipitate was formed in the methyl alcoholic solution, both the ammonia and the alcohol were driven off under reduced pressure. The viscous residue when redissolved in ethyl alcohol and precipitated with ether formed an amorphous opalescent deposit which retained about 1 molecule of ethyl alcohol. After standing several weeks in the refrigerator, spherical aggregates of hard yellow crystals were obtained from the mother liquor. They melted at 234°C . (corrected) and gave the following analytical values.

0.1060 gm. substance:	0.1812 gm. CO_2 and 0.0524 gm. H_2O .
0.0948 " " "	: 0.1604 " " " 0.0482 " "
0.0980 " " "	required (Kjeldahl) 12.25 cc. 0.1 N acid.
$\text{C}_{12}\text{H}_{16}\text{O}_6\text{N}_4$ (312.16).	Calculated. C 46.14, H 5.16, N 17.95.
	Found. " 46.61, " 5.53, " 17.50.
	" " 46.14, " 5.58.

The rotation was

$$[\alpha]_{\text{D}}^{25} = \frac{-0.39^{\circ} \times 100}{2 \times 0.925} = -21^{\circ} \text{ (in ethyl alcohol)}$$

$$[\alpha]_D^{25} = \frac{-0.38^\circ \times 100}{2 \times 0.495} = -38^\circ \text{ (in 0.5 N NaOH)}$$

The rotation of a sample of dimethyl xanthosine from natural xanthosine was newly found to be

$$[\alpha]_D^{25} = \frac{-0.58^\circ \times 100}{2 \times 1.245} = -23^\circ \text{ (in ethyl alcohol)}$$

Comparison of the Rate of Hydrolysis.—Comparison of the rate of hydrolysis in the case of the theophylline riboside and in that of dimethyl xanthosine could not be carried out polarimetrically due to the small change in rotation. Because of the good checks obtained in the case of the xyloside by the use of the Shaffer titrimetric method (6), we followed the reaction by this method.

TABLE II.
Hydrolytic Action of 0.1 N HCl at 100°C.

Time.	Volume applied.	Pentose.	Hydrolysis.	$k \times 10^4$
On 1.85 per cent solution of theophylline riboside.				
<i>min.</i>	<i>cc.</i>	<i>mg.</i>	<i>per cent</i>	
120	0.2	0.335	18.7	7.50
On 2.595 per cent solution of theophylline riboside.				
60	0.4	0.55	11.0	7.81
120	0.4	0.88	17.6	7.00
240	0.2	0.79	31.6	6.87
On 2.565 per cent solution of dimethyl xanthosine.				
120	0.4	0.80	16.3	6.44
240	0.2	0.85	34.6	7.68

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SYNTHETIC NUCLEOSIDES.

II. SUBSTITUTED URACIL XYLOSIDES.

BY P. A. LEVENE AND HARRY SOBOTKA.

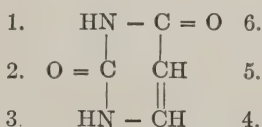
From the Laboratories of The Rockefeller Institute for Medical Research.)

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In the preceding paper the identity of a synthetic purine nucleoside with a derivative of a genuine nucleic acid was discussed. In the present paper we give details of a series of experiments carried out with derivatives of uracil.

Although Fischer successfully synthesized purine glucosides, he was less fortunate in his attempts at preparing the corresponding pyrimidine derivatives (1, 2). Neither from 4-methyluracil nor from cytosine, thiouracil, or ethyl thiouracil could substances be synthesized similar to the natural uridine and cytidine. Contrary to their prototype, all of the synthetic products reduced Fehling's solution. Their extreme instability makes it evident that the carbohydrate component is not linked with either of the nitrogen atoms.

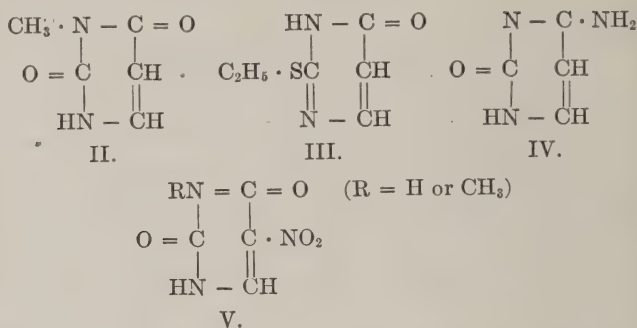
On comparing the formulæ for purine derivatives and for uracil, it is evident that in the ring of the latter derivative the chances for coupling with a non-enolizing imide group are not the same as in the case of theophylline.



I.

Through the alternation of CO and NH groups in the uracil ring there is always a twofold possibility for substitution, as is the case with theobromine and hydroxycaffeine. The difficulties in

controlling the course of substitution even in partially substituted uracils are seen from the following figures.



Substitution on carbon atoms (2) or (6) by monovalent group such as alkoxyl, mercapto (III), or amino (IV) groups prevent further reaction of the adjacent nitrogen; a replacement of the oxygen by the bivalent sulfur tends to promote enolization whereas substitution of two monovalent groups in the carbonyl group cannot be accomplished. In our attempt, we, nevertheless varied both the substituting groups and their positions over large range to cover any possible change in reactivity of the molecule.

Both *1-methyluracil* and *cytosine* (II and IV) were chosen for their free CO-NH group in position (2, 3).

The *5-nitrouracil* (V) which can be substituted in position (1, (2), (3), or (4) shows a remarkably strong acidic character which might possibly affect the relative reactivity of those positions.

In the *2-ethyl thio 6-oxy pyrimidine* (III), the free CO-NH group is placed in position (1, 6). The possibility is not excluded that an isomer exists having a double bond between atoms (1 and (2), which would permit substitution in position (3).

Finally, in the acidic *1-methyl-5-nitrouracil* (V; R = CH₃) replaceable hydrogen atoms are left in position (2) or (3).

Despite the varying character of the substituents, the reaction of acetobromopentose upon the silver compounds led to the formation of only 2- and 6-linkages; this was made evident by the readiness with which they underwent both acid and alkaline hydrolysis, the latter suggesting a close relationship to the class of imido esters. It is remarkable that cytosine did not react at a

Having failed to obtain substances possessing the same stability as genuine uridine we attempted to employ for synthesis the sodium and potassium salts in place of the silver salts of pyrimidines. T. B. Johnson and F. W. Heyl (3) have shown that whereas the silver compound of 2-anilidouracil reacts with methyl iodide to give only 6-methoxy-2-anilidouracil, the potassium salt gave a mixture of 6-methoxy and 1-methyl-6-hydroxy compounds. Although well defined potassium salts were prepared from the acidic 5-nitro- and 1-methyl-5-nitrouracil, only the undesired isomeric pentosides were formed.

From the above figures it is seen that the sugar group is attached in the different pentosides in the following way. In the 1-methyl-5-nitrouracil, in position (2); in the 2-ethyl thio compound, probably in position (6); in the 5-nitrouracil, the sugar group may enter in either position (2) or (6). The same possibility also exists in 1-methyl uracil. In the two latter instances, no crystalline products could be obtained, even when the potassium salt of nitrouracil was prepared from nitrouracil-4-carboxylic acid. This fact seems to indicate that mixtures of the two isomers were formed.

Rate of Hydrolysis of the Nucleosides.—The course of acid hydrolysis has been followed by the polariscopic method in view of the fact that boiling with Fehling's solution causes by itself complete hydrolysis of these nucleosides. All xylosides under investigation exhibit a dextrorotation, except the nitro compounds; in this point at least, one is reminded of the natural pyrimidine nucleosides. The mercapto compound resembles natural uridine (4) in that its dextrorotation is increased in acid solutions.

It may be mentioned that no rise in dextrorotation during the earlier stages of hydrolysis has been observed which fact indicates the absence of the α -xylosides. This corroborates the general rule that acetobromosugars always furnish β -glucosides.

On comparing the rates of hydrolysis, it was found that the 1-methyluracil xyloside was relatively stable.¹ No change in rotation took place in 0.2 N HCl within 3 hours at the temperature of the boiling water bath. N acid under these conditions brings about 90 per cent hydrolysis.

¹ The methyl compound shows a slightly higher stability also on boiling in alkaline copper solution than do the other derivatives.

Nitrouracil, though unaltered by 0.2 N acid in the cold, undergoes approximately 4 per cent hydrolysis within 2 hours at 100°. Its acetyl compound is even less stable, for it is hydrolyzed 70 per cent after 1 hour under the same conditions.

The mercapto compound shows still greater instability. After the initial immediate increase in rotation with 0.2 N hydrochloric acid in the cold, a slow drop in rotation is to be observed which becomes constant after 2 hours but on heating the solution a further drop takes place and hydrolysis is complete after another 2 hours. We do not attribute this effect to the existence of two compounds of different stability for even the second stage of hydrolysis is far too rapid to indicate a nitrogen linkage. The strong odor of mercaptane already developed with cold acid gives a clew to the first drop in rotation, which might be explained by the elimination of the mercapto group.

From these observations the possibility suggests itself that linkage in position (6) is less stable than the one in position (2).

The essential differences in the stability of the nucleosides synthesized by us, and of the natural nucleosides, and furthermore, the successful efforts of synthesis with theophylline, which does not permit of an oxygen linkage, corroborates the uridine formula which has been given by one of us in 1913 (5) and which recently has been confirmed from quite another point of view (6).

EXPERIMENTAL PART.

*Reaction between Acetobromoxylose and Silver Compounds of Uracil Derivatives.*²—Silver compounds from uracil and its derivatives are obtained by mixing their hot dilute aqueous solutions with the calculated amount of dilute silver nitrate solution. The liberated nitric acid is carefully neutralized by ammonia. The white precipitates are subsequently washed with water, alcohol, and ether and should be protected from the sunlight. Theoretical yields are obtained.

² The uracil derivatives have been prepared according to the directions given by H. L. Wheeler and H. F. Merriam (7) (2-mercaptouracil), T. B. Johnson and F. W. Heyl (3) (1-methyluracil), H. Biltz and M. Heyn (8) (5-nitrouracil), and R. Behrend and M. Lehmann (9) (1-methyl-5-nitrouracil).

Ethyl Thiouracil Silver. 0.1008 gm. substance: 0.0413 gm. Ag.

$C_6H_7ON_2SAg$ (263.02). Calculated. Ag. 41.02.

Found. " 40.96.

1-Methyluracil Silver. 0.1015 gm. substance: 0.0466 gm. Ag.

$C_5H_5O_2N_2Ag$ (232.94). Calculated. Ag. 46.31.

Found. " 45.91.

Cytosine Silver. 0.1002 gm. substance: 0.0493 gm. Ag.

$C_4H_4ON_3Ag$ (217.94). Calculated. Ag. 49.50.

Found. " 49.20.

These compounds were added in a fine powdered state to a solution of acetobromoxylose in xylene. Boiling under reflux condenser is continued until the liquid gives no more halogen reaction. The residue is separated and twice more extracted by means of hot xylene.

2-Ethyl Thiouracil Triacetyl Xylose.—The compound was thus prepared from 13 gm. of the silver salt of 2-ethyl thiouracil and 17 gm. of acetobromopentose. The xylene was removed from the filtrate under diminished pressure. By alternating recrystallization from ethyl and methyl alcohol 11 gm. (55 per cent of the theory) of the substance melting at 104–105°C. (all melting points corrected) were obtained.

0.1000 gm. substance: 0.1790 gm. CO_2 and 0.0478 gm. H_2O .

0.1094 " " : 6.20 cc. nitrogen gas at 24°C. and 768.9 mm.

$C_{17}H_{22}O_8N_2S$ (414.26). Calculated. C 49.24, H 5.35, N 6.76.

Found. " 48.81, " 5.03, " 6.59.

$$[\alpha]_D^{25} = \frac{+1.36^\circ \times 100}{1 \times 4.795} = +28.4^\circ$$

Partial hydrolysis of this compound by methyl alcoholic ammonia led to the formation of *ethyl thiouracil xyloside* which, after removal of the ammonia and most of the alcohol, crystallized. Recrystallization from ethyl alcohol yielded white needles, fairly soluble in water. Melting point 114–115°C.

0.0958 gm. substance: 0.1594 gm. CO_2 and 0.0482 gm. H_2O .

0.0932 " " : 7.60 cc. nitrogen gas at 24°C. and 762.8 mm.

$C_{11}H_{16}O_5N_2S$ (288.15). Calculated. C 45.81, H 5.60, N 9.72.

Found. " 45.37, " 5.63, " 9.40.

The rotation in methyl alcohol was

$$[\alpha]_D^{25} = \frac{+1.20^\circ \times 100}{1 \times 5.58} = +21.5^\circ$$

The substance is very unstable towards alkali. 2 minutes boiling with Fehling's solution released 55 per cent of the reducing sugar.

By addition of 0.1 cc. of concentrated HCl to 3 cc. of the solution in methyl alcohol, the rotation increased immediately to

$$[\alpha]_{\text{D}}^{25} = \frac{+1.87^{\circ} \times 100}{1 \times 5.40} = +34.6^{\circ}$$

A solution in 0.2 N HCl decreased from the initial rotation of 0.73° during a few hours to 0.51° . At this point it stopped, but by heating to 100°C . for 2 hours, the value calculated for equilibrium xylose was attained, the pyrimidine base itself having no optical activity.

$$[\alpha]_{\text{D}}^{25} = \frac{+0.73^{\circ} \times 100}{1 \times 2.30} = +31.7^{\circ}$$

Final value found 0.18° ; the calculated rotation for a 1.20 per cent solution of xylose would be

$$\alpha_{\text{D}} = \frac{+19^{\circ} \times 1.20}{1 \times 100} = +0.23^{\circ}$$

9 gm. of acetobromoxylose and 6 gm. of 1-methyluracil silver yielded 4 gm. of *1-methyluracil xylose triacetate* which settled down from the xylene solution. (Neither from the mother liquor nor from further extracts of the silver bromide residue could more of the product be obtained. Here as well as in the following experiments only amorphous masses could be extracted, the high nitrogen content of which despite repeated reprecipitations indicated that they consisted chiefly of uncombined base.)

0.1019 gm. substance: 0.1874 gm. CO_2 and 0.0486 gm. H_2O .

$\text{C}_{16}\text{H}_{20}\text{O}_9\text{N}_2$ (384.18). Calculated. C 49.98, H 5.25.

Found. " 50.14, " 5.33.

In 2 per cent solution in the 2 dm. tube no optical activity was detected. By removal of the acetyl groups, the *1-methyluracil xyloside* was formed which reduced Fehling's solution like the mercapto compound.

0.0993 gm. substance: 0.1662 gm. CO_2 and 0.0512 H_2O .

0.1000 " " required (Kjeldahl) 8.55 cc. 0.1 N acid.

$\text{C}_{10}\text{H}_{14}\text{O}_6\text{N}_2$ (258.13). Calculated. C 46.49, H 5.47, N 11.37.

Found. " 45.63, " 5.76, " 11.97.

This substance had the following rotation in methyl alcohol.

$$[\alpha]_{\text{D}}^{25} = \frac{+0.56^{\circ} \times 100^{\circ}}{1 \times 2.05} = +27.3^{\circ}$$

Neither at normal temperature nor at 100°C. were the substance and its optical activity affected by 0.2 N HCl within 3 hours. At least N acid acting on the xyloside in 1.17 per cent concentration for 3 hours at 100°C. caused a drop in rotation from 0.64° to 0.30° (2 dm.), the final value for 100 per cent liberated xylose figuring at 0.26°.

5-Nitrouracil Acetyl Xyloside.—5-Nitrouracil silver reacted with the acetobromo sugar to form an ochre-colored product, which was but slightly soluble in xylene. The yield was increased by alcoholic extraction of the solid residue. The substance could not be purified by recrystallization for its solutions in methyl alcohol underwent spontaneous decomposition above 60°C., apparently due to a reaction between NO₂ group and sugar radicle.

The same difficulty arose on deacetylation, unless the reaction was carried out with small quantities. The *5-nitrouracil xyloside* despite repeated recrystallization from ethyl alcohol and ethyl acetate could not be obtained in an analytically pure state. It reduces, however, larger amounts of alkaline copper solution than could be explained by assuming a contamination with uncombined xylose.

$$[\alpha]_{\text{D}}^{25} = \frac{-0.22^{\circ} \times 100}{2 \times 6.12} = -1.80^{\circ}$$

After 2 hours boiling with 0.2 N HCl the rotation increased to -0.18°; after 4 hours, to -0.15°, corresponding to 4 and 7 per cent hydrolysis respectively. (Final value +0.82°.)

Cytosine silver, with acetobromoxylose, did not form a condensation product containing nitrogen. Despite the precautions against moisture taken throughout these experiments in the case of cytosine considerable quantities of *triacetyl xylose* crystallized from the xylene, forming spherical aggregates almost invisible in the solvent by their similar index of refraction. There was no sharp melting point as the material was an α , β equilibrium mixture.

0.1063 gm. substance: 0.1820 gm. CO₂ and 0.0554 gm. H₂O.

C₁₁H₁₆O₈ (276.13). Calculated. C 47.80, H 5.84.

Found. " 47.90, " 5.99.

$$[\alpha]_D^{25} = \frac{+5.24^\circ \times 100}{2 \times 4.976} = +52.65^\circ \text{ (water; final value)}$$

In other instances, however, where no synthesis was obtained, considerable quantities of acetobromo sugar were recovered in crystalline state, thus showing the absolute absence of water.

Alkali Compounds of Uracils.—These were obtained by dissolving the uracil derivatives in aqueous or alcoholic alkali hydroxide. They are extremely soluble and by evaporation form a syrup and finally hard crusts. Although a slight excess of alkali was used, the values for Na or K were always found to be too low. Even the mercaptouracil potassium which crystallized from concentrated solution, in long silk-like needles, after drying under vacuum at the temperature of boiling xylene retained considerable amounts of water, which was fatal for the condensation with acetobromo sugar.

Both the sodium and potassium salts of ethyl thiouracil in xylene as well as in absolute ethyl alcohol failed to react with the sugar but left residues of the unchanged base. Besides, white platelets were precipitated in one instance from ethyl acetate with petroleum ether, which analyzed for an addition product C₆H₈O₂N₂S·HBr.

No satisfactory results could be obtained with the potassium salt of 5-nitrouracil, prepared from the 5-nitrouracil-4-carbonic acid by heating its potassium salt to 170°C. The amorphous product had a specific rotation of about -50°. The optical activity was lost by heating 3 hours with 0.2 N acid to 100°C., due to hydrolysis and possibly decomposition of the sugar by freed nitric acid.

The beautiful slightly green potassium salt of 1-methylnitrouracil, when brought in contact with the acetobromoxylose yielded greenish crystals, which melted at 243°C. (corrected) and analyzed for methyl nitrouracil triacetyl xyloside.

0.0980 gm. substance: 0.0424 gm. K₂SO₄.

C₆H₄O₄N₃K (209.16). Calculated. K 18.69.

Found. " 18.25.

0.1006 gm. substance: 0.1640 gm. CO₂ and 0.0416 gm. H₂O.

0.1000 " " : 8.90 cc. nitrogen gas at 25°C. and 764.5 mm.

C₁₆H₁₉O₁₁N₃ (429.18). Calculated. C 44.74, H 4.46, N 9.79.

Found. " 44.45, " 4.62, " 10.24.

This substance also reduces Fehling's solution. It is almost insoluble in water and alcohol and had the following rotation in pyridine methyl alcohol 1:1.

$$[\alpha]_D^{25} = \frac{-0.76^\circ \times 100}{2 \times 0.836} = -45.5^\circ$$

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CHEMICAL FINDINGS IN THE BLOOD OF THE NORMAL DOG.

BY RUSSELL L. HADEN AND THOMAS G. ORR.

(From the University of Kansas School of Medicine, Kansas City.)

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In experimental work on the dog involving chemical studies of the blood, it is essential to know the average content and the range of the various components in the normal animal. Several observers (1) have reported such data with rather widely different results. Usually only a relatively few determinations have been made.

We have estimated the non-protein nitrogen, the urea nitrogen, the amino acid nitrogen, the creatinine, the chlorides, the sugar, and the CO₂-combining power on a series of dogs sufficiently large to allow determination of the normal range of these components. The results represent consecutive determinations made by a uniform technique.

Method.

Usually the animals have been fasted for 48 hours before the specimens were obtained. In some instances the dogs were bled the morning after they were received in the laboratory. The specimens for analysis were obtained from the jugular vein with a syringe, 20 cc. of blood were withdrawn and immediately run into a tube containing 4 drops of a saturated solution of potassium oxalate.

The protein-free filtrates have been made for the most part by a modification of the Folin-Wu technique described by one of us (2). The adoption of this procedure represents the only change in method made during the time the analyses were being made. The non-protein nitrogen, the sugar, and the creatinine have been determined by the method of Folin and Wu (3), the CO₂-combining power by the method of Van Slyke and Cullen (4),

TABLE I.
Chemical Findings in the Blood of the Normal Dog.

Determinations,	No. of dogs.	Amount per 100 cc.											
		Average.	Highest.	Lowest.	Distribution.								
		mg.	mg.	mg.	Below 20 mg.		20-30 mg.		30-40 mg.		Over 40 mg.		
Non-protein nitrogen.....	200	30.8	78.0	16.1	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	
					2	1	114	57	61	30.5	23	11.5	
					Below 7 mg.		7-12 mg.		12-17 mg.		Over 17 mg.		
Urea nitrogen..	200	11.7	42.1	5.1	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	
					12	6	117	58.5	49	24.5	22	11	
					Below 1 mg.		1.0-1.5 mg.		1.5-2.0 mg.		Over 2.0 mg.		
Creatinine.....	200	1.5	2.6	0.5	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	
					3	1.5	95	47.5	92	46	10	5	
					Below 5 mg.		5-7 mg.		7-9 mg.		Over 9 mg.		
Amino acid nitrogen.....	100	6.7	14.6	2.0	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	
					5	5	65	65	22	22	8	8	
					Below 60 mg.		60-80 mg.		80-100 mg.		Over 100 mg.		
Sugar.....	200	82	152	35	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	
					25	12.5	67	33.5	85	42.5	23	11.5	
					Below 440 mg.		440-470 mg.		470-500 mg.		Over 500 mg.		
Chlorides (as NaCl).....	200	468	640	340	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	
					29	14.5	96	48	54	27	21	10.5	

TABLE I—*Concluded.*

Determinations.	No. of dogs.	Amount per 100 cc.										
		Average.	Highest.	Lowest.	Distribution.							
		mg.	mg.	mg.	Below 25 vol. per cent.		25-35 vol. per cent.		35-45 vol. per cent.		Over 45 vol. per cent.	
					No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
CO ₂ -combining power (plasma).....	200	34.8	56.0	17.4	21	10.5	80	40	75	37.5	24	12

the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (5), the amino acid nitrogen by the method of Folin (6), and the chlorides on the tungstic acid filtrate in the manner suggested by Gettler (7).

Observations.

The results of the determinations are shown in Table I. The average for each component is practically the same as that accepted as normal for the blood of man except the CO₂-combining power, which is lower.

SUMMARY.

The results of 200 consecutive analyses of the blood of the normal dog are reported.

The average non-protein nitrogen is 30.8 mg. per 100 cc.; urea nitrogen, 11.7 mg.; creatinine, 1.5 mg.; amino acid nitrogen, 3.7 mg.; sugar, 82 mg.; chlorides (as NaCl), 468 mg.; and the CO₂-combining power of the plasma, 34.8 volumes per cent.

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THE CONCENTRATION OF VITAMIN B. II.

BY P. A. LEVENE AND B. J. C. VAN DER HOEVEN.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 30, 1925.)

In a previous publication¹ it was shown that active growth-promoting material contained in brewers' yeast consisted of a mixture of organic and mineral substances even after it was concentrated to such a degree that quantities of it containing 0.06 mg. of nitrogen per day were sufficient to promote growth. It was then realized that further progress of the work would depend in a large degree on the reduction of the losses associated with the process of concentration. It was therefore attempted to improve every phase in the preparation of the material. The starting point of our work was the fraction precipitable by 80 per cent alcohol and prepared in a general way according to directions of Osborne and Wakeman and referred to in this communication as the "O. W." fraction.² In the earlier work a quantity of this material, containing 3.0 mg. of nitrogen, was the minimum required to promote growth. This material was now thoroughly shaken with absolute alcohol until it acquired the character of a very fine powder which, on drying under reduced pressure, was no longer hygroscopic. The material prepared in this manner was active in quantities of from 10 to 15 mg. (containing 1.0 to 1.5 mg. of nitrogen) per day.

Precipitation with Basic Lead Acetate.

The first phase in the purification of this material in the earlier work consisted in precipitation by means of barium hydroxide. It was now found advantageous to add as an intermediary step the precipitation of the active substance by means of basic lead acetate.

¹ Levene, P. A., and van der Hoeven, B. J. C., *J. Biol. Chem.*, 1924, lxi, 429.

² Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

Lead acetate had been applied for this purpose on previous occasions, but unsuccessfully. The cause of the failure lay in the method which was employed for the liberation of the active material from the lead precipitate; at that time, hydrogen sulfide was used for the removal of the lead and no effective way was found to recover the active principle from the sulfide of lead.

It was now found that when the lead was removed by means of sulfuric acid, the active principle could be recovered practically quantitatively. By this step alone, the potency of the product increased three to four times.

To this material the precipitation with barium hydroxide was applied in the manner described in the first paper. By this treatment a product was obtained which was approximately as active as the one previously prepared through the silica adsorption process. The new product was active in quantities containing from 0.10 to 0.20 mg. of nitrogen.; *i.e.*, in quantities of 2.0 to 4.0 mg. of the substance. The substance contained 5.2 per cent of nitrogen, calculated ash-free. From this it is seen that by the lead-barium precipitation a considerable quantity of inert nitrogenous material was removed, since this product contained less nitrogen than the starting material. The yield of this material was between 30 and 40 per cent.

Extraction with Silica Gel.

Further purification was again accomplished by extraction with silica gel and, as will be seen later, a selective purification was accomplished by this adsorbent. The adsorbed material was removed from the silica twice by alcohol, acidulated to pH 3. These extracts contained materials of only moderate potency. A third extraction was made at a pH of 9. This extract contained the most potent material thus far prepared. It was active in about 0.1 mg. of substance, containing 0.015 mg. of nitrogen. In view of the fact that the absolute volume of the solution and hence the proportions of acid and base used in the experiments were large in proportion to the material, it was thought advantageous to employ an acid of which the salts would be soluble in alcohol. Since it was found that the active principle is not soluble, it could thus be separated from the mineral impurities. With this aim in view, hydriodic acid was employed for the extraction of

the silica. Nevertheless, the product obtained from the silica extract by alcohol precipitation contained about 80 per cent of ash, though the material contained only traces of iodine. The substance dried in the desiccator (under diminished pressure) contained 3.1 per cent of nitrogen or 15 per cent calculated for the dry ash-free material. It contained 3.4 per cent of inorganic phosphorus and 4.6 per cent of total phosphorus. It gave negative tests for protein (Biuret and Millon's) and positive for pentoses. On hydrolysis the product reduced Fehling's solution. From all these data it is easily concluded that the substance was still of a very complex nature. The yield of this material was about 35 per cent of the lead and barium product, or between 3 and 5 per cent of the Osborne-Wakeman fraction.

Attempts to Remove the Mineral Constituents of the Active Principle.

The great disturbing fact in the purification of the growth-promoting principle by means of adsorbents is the coadsorption and even introduction of mineral impurities. Of these, the salts of phosphoric acid are particularly disturbing. These can be removed from the lead and barium product by precipitating the active material with absolute alcohol from strongly acid solution (pH 2). By precipitating under these conditions the sulfuric acid extract of the barium precipitate, a substance was obtained which was potent in quantities of about 2.5 mg. (containing 0.10 mg. of nitrogen) per day. This product contained only 6.1 per cent of mineral impurity. It contained no phosphorus. It had the following composition. C = 40.0 per cent; H = 5.8 per cent; N = 4.1 per cent. The substance gave a strong orcinol test and on hydrolysis reduced Fehling's solution. It is thus seen that in the main, this product consisted of carbohydrates. Whether the carbohydrate is an integral part of the active principle or is only an inert admixture cannot be said.

A material still more potent and apparently of the same composition was obtained when the lead-barium product containing the mineral impurities was dissolved in a minimal amount of hydrochloric acid of specific gravity 1.19 and to the solution enough 8 per cent alcohol was added to make the alcohol concentration 10 per cent. This material still contained about 4 per cent of nitrogen and was active in daily doses of 1.25 mg.

However, it was found that the material purified in this manner was not as readily further improved by silica adsorption as was the phosphorus-containing material. It was stated above that the product obtained through silica adsorption from the latter material was potent in daily doses of 0.10 mg. and contained 15 per cent, whereas by the same treatment of the purified product, a substance was obtained which contained only 4.2 per cent of nitrogen and which was active in doses of 0.6 mg. per day. Thus it seems as if the phosphorus-containing impurity is essential for the selective adsorption by silica.

EXPERIMENTAL PART.

Precipitation with Lead and Barium.

250 gm. of purified Osborne and Wakeman's fraction are dissolved in water, centrifugalized after one night's standing in a cold room, and precipitated with 5 liters of basic lead acetate solution. The precipitate collected by centrifugalization is treated with excess 10 per cent sulfuric acid until it reacts acid to Congo paper and is then centrifugalized. To the clear solution are added 8 liters of a cold saturated barium hydroxide solution sufficient to make it alkaline. The precipitate is rapidly centrifugalized and taken up in 10 per cent sulfuric acid. The filtrate from the barium sulfate is used for further purification.

Lead-Barium Precipitation.

O.W. Fraction.

(1.2) 10 gm. N

[8000] units

Precipitated basic lead acetate.

Filtrate No. 145

(2.2) 3.8 gm. N

= [1500] units

Precipitate No. 146

(0.3) 2.3 gm. N

= [7000] units

Precipitated barium hydroxide.

Filtrate No. 147

(1) 1.6 gm. N

= [1600] units

Precipitate No. 148

(0.15) 0.5 gm. N

= [3300] units

O.W. Fraction.

(1.5) 3 gm. N[2000] units

With basic lead acetate.

Filtrate No. 123

(.4) 2.3 gm. N=[600] units

Precipitate No. 124

(0.5) 1.00 gm. N=[2000] units

With barium hydroxide.

Filtrate No. 125

(0.8) 0.81 gm. N=[1000] units

Precipitate No. 131

(0.15) 0.090 gm. N=[600] units*Extraction with Silica Gel.*

A quantity of solution corresponding to 250 gm. of the O.W. fraction is brought to pH 5 and kept at that for $\frac{1}{2}$ hour with continuous stirring and addition of 500 gm. of silica gel. The silica is then filtered off, suspended in 3 liters of 30 per cent alcohol, kept at pH 3 (with HI), filtered, this last process repeated once more, and finally the silica is suspended in 3 liters of distilled water, stirred, and kept at pH 9 for $\frac{1}{2}$ hour. The clear solution obtained from this extraction is immediately neutralized and evaporated to a small volume, the silicic acid removed, and the substance (5 cc.) precipitated by 250 cc. of absolute alcohol.

Extraction with Silica Gel.

Lead-barium precipitate.

(0.15) 1.60 gm. N[11,000] units

Extracted with 500 gm. silica at pH 5.

Filtrate No. 174

(0.20) 1.31 gm. N=[6500] units

1. Extract (pH 3) No. 175

(0.09) 0.12 gm. N=[1300] units

2. Extract (pH 3) No. 176

(0.08) 0.05 gm. N=[600] units

3. Extract (pH 9) No. 177

(0.015) 0.02 gm. N=[1400] units

Experiments with Substances from Which the Phosphates Had Been Removed.

No. 393 (Lead-barium precipitate).

(0.17) 0.070 gm. N[400] units

With absolute alcohol at pH 2.

Filtrate No. 394

(0.20) 0.045 gm. N=[220] units

Precipitate No. 395

(0.10) 0.020 gm. N=[200] units

No. 417 (Phosphate-free).

(0.12) 0.020 gm. N[160] units

With HCl of sp. gr. 1.19, and 70 per cent alcohol.

Filtrate No. 419

(0.15) 0.012 gm. N=[80] units

Precipitate No. 420

(0.05) 0.0042 gm. N=[80] units

No. 395 and following (Phosphate-free).

(0.15) 0.060 gm. N[400] units

Extracted with 500 gm. silica gel at pH 5.

Filtrate No. 440

(0.35) 0.050 gm. N=[140] units

1. Extract (pH 3) No. 441

(Inactive) 0.002 mg. N[0] units

3. Extract (pH 9) No. 442

(0.025) 0.04 mg. N[160] units

Analytical Data.

Substance.	N in ash-free sub- stance.	C	H	Activity.	Qualitative reactions.
	per cent	per cent	per cent	mg. N	
Osborne-Wakeman fractions.	10.1			1.0-1.5	
Lead-barium precipitate.....	5.2			0.10	
Silica extract from lead- barium precipitate.....	15			0.015	Molisch + Millon — Biuret —
Lead-barium precipitate, phosphate-free.....	4.1	40.0	5.8	0.10	Orcinol + Biuret — Fehling +
Silica extract from lead- barium precipitate, phos- phate-free.....	4.2			0.025	

CONCLUSIONS.

1. The Osborne and Wakeman concentrate of the yeast extract can be further concentrated.
2. By successive precipitation, first by lead acetate and second by barium hydroxide, a product is obtained which contains 5.2 per cent of nitrogen (calculated for the ash-free substance) and is potent in daily doses of from 2.0 to 4.0 mg. per day.
3. The ash-free product from the material described in (2) has an elementary composition approaching that of carbohydrates. It yields on hydrolysis reducing sugars, contains about 4 per cent of nitrogen, and is potent in daily doses of 1.25 mg. per day.
4. From the material described in (2) by silica adsorption a product is obtained which is potent in daily doses of 0.100 mg. and which contains 15 per cent of nitrogen.
5. From the product described in (3) by the silica treatment a material is obtained which is potent in daily doses of 0.600 mg. and which contains only 4 per cent of nitrogen.

STROPHANTHIN.

VIII. THE CARBONYL GROUP OF STROPHANTHIDIN.

BY WALTER A. JACOBS AND ARNOLD M. COLLINS.

From the Laboratories of The Rockefeller Institute for Medical Research.)

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The presence of olefinic groups in dianhydrostrophanthidin has been demonstrated by previous hydrogenation experiments¹ and it was hoped that these double bonds might be made the point of attack in oxidation experiments with the formation of substances, either degradation products or those which would give information as to the position of the double bonds. However, as we have previously noted this substance exhibits towards permanganate in acetone solution no greater degree of unsaturation than strophanthidin itself. In agreement with this observation oxidizing agents as employed in the present experiments have given substances in which the double bonds have resisted oxidation and in which the carbonyl group instead has been the point of attack. But a study of these substances has permitted the drawing of definite conclusions regarding the nature of the carbonyl group in strophanthidin and its derivatives.

Although no positive evidence was previously available the carbonyl group has been referred to as probably ketonic in character. This was inferred mainly from the behavior of strophanthidin and its derivatives towards aldehyde reagents. Strophanthidin, dihydrostrophanthidin, and isostrophanthidin do not reduce Fehling's solution. Although strophanthidin itself reduces Tollens' reagent this property we now know is referable to the unsaturated lactone group since it is lost by conversion into the dihydro derivative. Likewise, isostrophanthidin only very slowly reduces this reagent. An aromatic aldehyde group seemed out of the question although a tertiary aliphatic

¹ Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxxiii, 123.

aldehyde might still have permitted the above behavior. But in the deportment of these substances towards alkali, no positive evidence of a Cannizzaro reaction was obtained. By the action of alcoholic alkali it was possible to convert strophanthidin quantitatively into isostrophanthidin although boiling alkali was found to give rise to obscure, highly colored, amorphous alteration products. The present studies, however, on the behavior of dianhydrostrophanthidin, strophanthidin, and dihydrostrophanthidin towards oxidizing agents, appear to demonstrate definitely the aldehydic character of the carbonyl group.

When dianhydrostrophanthidin was oxidized in acetic acid solution with chromic acid a neutral crystalline substance, $C_{23}H_{26}O_4$, was the principal reaction product which formed neither an oxime nor an acyl compound. Its behavior towards boiling alkali demonstrated it to be a dilactone. Both lactone groups were easily saponified to a dibasic acid, $C_{23}H_{30}O_6$, which showed little tendency to lactonize again. The dilactone still reduced Tollens' reagent, showing the retention of the original unsaturated lactone group. The new lactone group was formed presumably by direct oxidation of the oxidic form of dianhydrostrophanthidin since the dilactone when once opened showed little tendency to close again, at least under conditions which resembled those which obtained during the oxidation. However, a very small amount of an acid by-product was isolated from the reaction mixture which possessed the composition, $C_{23}H_{28}O_5$, and proved to be a lactone acid. The lactone group was that originally present in dianhydrostrophanthidin and the acid group was formed by direct oxidation of the aldehydic form. When saponified this acid gave the above described dibasic acid. In other words either dianhydrostrophanthidin in acetic acid solution exists as an equilibrium mixture between the oxidic and aldehydic forms in which the former apparently preponderates; or, as appears more likely, the chromic acid acts far more specifically upon the oxidic form so that the equilibrium of the mixture is constantly shifting to the oxidic form as the latter is disposed of by the oxidizing agent. Potassium permanganate was also found to exhibit a specific behavior towards dianhydrostrophanthidin in acetone solution. Although most of the substance was recovered unchanged the only crystalline oxidation product which

were made with the hexahydrodilactone and in this case it was possible to ascertain, as seemed *a priori* likely, that the refractory lactone group was that associated with the tetrahydrogenated double bonds. When only one lactone group was opened with a weak alkali, reacidification resulted in the recovery of the original hexahydrodilactone. Under the same conditions the tetrahydrodilactone yielded a lactone acid. This conclusion is supported by the fact that the dihydrogenated lactone group of dihydrostrophanthidin and its derivatives, as previously noted, relactonizes very readily after saponification.²

In view of the oxidation of the aldehydic group of dianhydrostrophanthidin to the lactone acid we have returned to a consideration of our former experience with strophanthidin itself.³ This substance in acetone solution had yielded with permanganate a substance which was described as a lactone acid, $C_{23}H_{30}O_7$, in which in some apparently obscure way the carbonyl group had been changed to carboxyl with simultaneous loss of two H atoms. This formula has now been shown to be incorrect and the acid there described possesses instead the formula $C_{23}H_{32}O_7$. The former uncertainty was largely due to the difficulty of drying without decomposition for analysis the substance which crystallized with water of crystallization. It is formed by the oxidation of the aldehydic group of strophanthidin to carboxyl. This was substantiated by the formation of the analogous dihydrolactone acid, $C_{23}H_{34}O_7$, by oxidation of dihydrostrophanthidin with permanganate both in acetone solution and after saponification in aqueous solution. This last procedure was inapplicable in the case of strophanthidin itself because of isomerization to isostrophanthidin. With the idea of showing the relationship of these acids to those obtained from dianhydrostrophanthidin an attempt was made to dehydrate them with concentrated hydrochloric acid. It was surprising, however, that instead of the expected anhydrolactone acids or perhaps anhydrodilactones neutral substances were obtained which differed from the original acids by only 1 mol of water. In other words the acids, $C_{23}H_{32}O_7$ and $C_{23}H_{34}O_7$, obtained respectively from strophanthidin and dihydrostrophanthidin, were converted into the neutral dilactones, $C_{23}H_{30}O_6$ and $C_{23}H_{32}O_6$.

² Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxiv, 383.

³ Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 556.

The former retained the power to reduce Tollens' reagent and the latter did not. These dilactones curiously enough exhibited behavior towards alkali similar to that shown by the tetrahydro- and hexahydrodianhydrodilactones in that the newly formed lactone group proved to be much more resistant to alkali than that originally present.

In view of the comparative rigidity of this second lactone or oxidic structure in the saturated substances a clue was obtained as to the nature of the so called pseudostrophanthidin described in a previous communication⁴ and which was formed by the action of strong hydrochloric acid on strophanthidin. This appeared to represent a stable oxidic form of the hydroxyaldehyde since it did not form an oxime. The unsaturated lactone group was demonstrated to be still intact by its behavior towards Tollens' reagent. Definite proof of the oxidic form was obtained by oxidation. Although permanganate in acetone solution was practically without action upon pseudostrophanthidin it was easily oxidized by chromic acid. Instead, however, of the expected dilactone, $C_{23}H_{30}O_6$, identical with that described above, a neutral substance, $C_{23}H_{28}O_6$, was obtained which was also found to contain two lactone groups. Further inquiry showed that the oxidation had simply proceeded a step further with oxidation apparently of one of the hitherto dormant hydroxyl groups to a ketone. The same ketodilactone was obtained by further oxidation with chromic acid of the above dilactone, $C_{23}H_{30}O_6$, obtained from strophanthidin.

The experience has thus shown rather a sharp contrast between the behavior of permanganate and chromic acid as oxidizing agents. The former appears to have acted specifically by oxidizing directly the aldehydic form to the acid. The previously described oxidation of isostrophanthidin or at least of isostrophanthidic acid to isostrophanthic acid with permanganate belongs unquestionably in this category. On the other hand, chromic acid appears to be specific for the conversion of the oxidic form of the aldehyde into the lactone.

Although the experience given here permits the coordination of a number of observations which have been made in the study

⁴ Jacobs and Collins,¹ p. 131.

of strophanthidin and its derivatives a number of uncertainties still persist. Such questions as the bearing of the rigidity of the saturated oxidic forms on structure and the reason for the failure of pseudostrophanthidin, and the lactone acid, $C_{23}H_{32}O_7$, and other derivatives which still possess the unsaturated lactone ring of strophanthidin, to isomerize to iso compounds under the influence of alkali remain to be answered. Again, the apparent failure of the acid, $C_{23}H_{32}O_7$ (then erroneously described as $C_{23}H_{30}O_7$), to react with hydroxylamine after saponification will require further study. These and other questions are at present under investigation.

Since pseudostrophanthidin and the dilactone, $C_{23}H_{30}O_6$, yield a ketodilactone, it would seem that at least one of the hydroxyl groups originally present must be secondary. Since in all likelihood the saturated lactone group in this compound is γ -oxidic and the same hydroxyl is presumably involved in the ring closure as that which is concerned in the oxidic forms of the anhydrostrophanthidins and which in the aldehydic form can be acylated, doubt is cast upon the previous interpretation which attributed to the remaining hydroxyls of strophanthidin a tertiary character. This conclusion was originally based on the failure to acylate more than one hydroxyl group. The possibility now appears that strophanthidin may have at least two free secondary hydroxyl groups. Further work may demonstrate all of them to be secondary, in which case strophanthidin will begin to assume the appearance of a complex molecule with a carbohydrate side chain. Such speculations must, however, await the accumulation of further data. Interesting in connection with the observations with these substances is the fact that all of the neutral hydroxy compounds still possess an appreciably bitter taste although not quite as marked as in the case of strophanthidin. These substances may also exhibit interesting physiological behaviors. This study is contemplated.

EXPERIMENTAL.

Oxidation of Dihydrostrophanthidin and Strophanthidin.

Lactone Acid, $C_{23}H_{34}O_7$ —3 gm. of dihydrostrophanthidin were shaken at ordinary temperature in 30 cc. of N sodium hydroxide

solution. Solution was complete within 10 minutes. After diluting to 100 cc. the mixture was oxidized at 10° with 17.5 cc. of 5 per cent permanganate or an amount slightly in excess of that required for 1 mol of O. The reagent was at first fairly promptly consumed, but towards the end the permanganate color persisted for some time. The filtrate was carefully acidified with Congo red with hydrochloric acid and, on rubbing, the lactone acid gradually separated as a sandy powder which consisted of small prisms and stout, pointed platelets. The mother liquor yielded an additional amount when concentrated at room temperature. It was found, however, advisable to neutralize the solution before concentration and then to reacidify to Congo red since the acid proved to be sensitive to a too vigorous treatment with mineral acid and was readily converted into amorphous material (partial lactonization). The total yield was 2.2 gm. The acid when recrystallized by careful dilution of a concentrated alcoholic solution slowly separated as a crust of short, stout platelets which contained 2 mols of water of crystallization. It slowly froths up when heated to $132\text{--}133^{\circ}$ and is easily soluble in alcohol and acetone and not appreciably soluble in the water-miscible solvents. In sulfuric acid it forms at first a brown-range color which deepens to a very characteristic deep purple-red.

$$[\alpha]_D^{25} = +47^{\circ} \text{ (} c = 1.015 \text{ in methyl alcohol for the hydrate).}$$

During the drying of the substance for analysis it appeared that slight decomposition occurred since the carbon figures were always too high. This recalls the experience with the analogous acid obtained from strophanthidin and also the experience with strophanthidin itself.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$\text{C}_{23}\text{H}_{34}\text{O}_7 \cdot 2\text{H}_2\text{O}$. Calculated. H_2O 7.86.

Found. " 7.88.

Anhydrous Substance.

$\text{C}_{23}\text{H}_{34}\text{O}_7$. Calculated. C 65.36, H 8.12.

Found. " 65.95, " 8.23.

In the above oxidation experiment the addition of permanganate sufficient to furnish 1 mol of oxygen was followed by the

further addition of four times that amount the permanganate was very slowly consumed. From the reaction mixture it was still possible to isolate a small amount of the above acid and no evidence was obtained of the formation of a ketonic acid analogous to that previously obtained by the oxidation after saponification of the lactone acid obtained from strophanthidin.⁵

The lactone acid with identical properties was also prepared although less conveniently by the use of permanganate in acetone solution in accordance with the procedure previously described for the preparation of the lactone acid from strophanthidin.

Air-Dry Substance. Found. H_2O 8.49, 8.19.

Anhydrous Substance. Found. (a). C 65.75, H 8.26.

(b). " 65.93, " 7.99.

0.1049 gm. of substance when titrated with 0.1 N NaOH against phenolphthalein required 2.30 cc. Calculated for 1 equivalent, 2.48 cc. After further addition of an excess of alkali the mixture was refluxed for 2 hours and again titrated. An additional 2.52 cc. were consumed.

Dilactone, $C_{23}H_{32}O_6$.—1.1 gm. of the above lactone acid were dissolved in 10 cc. of concentrated hydrochloric acid and the solution was kept in ice for 20 minutes during which it deepened to an olive color. Dilution with water caused the precipitation in small amount of yellow amorphous material, leaving a colorless solution after filtration. On standing the dilactone slowly crystallized as a sandy powder consisting of rosettes of microplatelets. When recrystallized from dilute alcohol it formed lustrous leaflets which contained 0.5 mol of water of crystallization. The substance melts at $232-234^\circ$ after preliminary softening and is insoluble in cold dilute alkali. It is easily soluble in acetone and chloroform and less readily so in alcohol. In sulfuric acid it gives on standing the characteristic red-purple color of the original acid. In dilute pyridine solution it does not reduce Tollens' solution. The substance possesses an appreciably bitter taste.

$$[\alpha]_D^{25} = +84^\circ \text{ (c = 1.000 in methyl alcohol).}$$

The two lactone groups exhibited a marked difference in their

⁵ Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 559.

resistance to the saponifying action of alkalies which was shown in the following titration experiments. 0.0618 gm. of anhydrous substance was boiled for 2 hours in 15 cc. of alcohol and 15 cc. of 0.1 N NaOH and titrated against phenolphthalein. Calculated for 1 equivalent, 1.53 cc. Found, 1.89 cc. 0.1085 gm. of substance was boiled for 3 hours in 15 cc. of 0.2 N NaOH. Calculated for 2 equivalents, 2.68 cc. Found, 2.24 cc. For complete saponification even longer boiling was apparently necessary.

Air-Dry Substance. Dried at 100° and 15 mm. over H₂SO₄.

C₂₃H₃₂O₆ · $\frac{1}{2}$ H₂O. Calculated. H₂O 2.18.

Found. " 1.98.

Anhydrous Substance.

C₂₃H₃₂O₆. Calculated. C 68.27, H 7.98.

Found. " 68.36, " 7.95.

Dilactone, C₂₃H₃₀O₆.—1 gm. of the lactone acid, C₂₃H₃₂O₇, which was obtained by oxidation of strophanthidin with permanganate in acetone solution, and which, in a previous communication,⁶ was given the incorrect formula, C₂₃H₃₀O₇, was converted into the dilactone in accordance with the procedure used above in the case of the dihydrolactone acid. After several days, crystallization began in the diluted reaction mixture. The separation was facilitated by salting-out with ammonium sulfate. Recrystallized from dilute alcohol it forms leaflets and long pointed platelets containing approximately 1 mol of water and is insoluble in alkali. It melts at 235–236° after preliminary sintering and, in sulfuric acid, gives at first an orange-brown color which changes to the characteristic purple-red color given by the original acid. It is soluble in acetone, chloroform, and alcohol. In dilute pyridine solution it reduces Tollens' solution.

$[\alpha]_D^{27} = +100^\circ$ ($c = 1.005$ in methyl alcohol).

Air-Dry Substance. Dried at 100° and 15 mm. over H₂SO₄.

C₂₃H₃₀O₆ · H₂O. Calculated. H₂O 4.28.

Found. " 3.38.

Anhydrous Substance.

C₂₃H₃₀O₆. Calculated. C 68.61, H 7.52.

Found. " 69.04, " 7.62.

0.0931 gm. of substance was boiled for 5 hours in 15 cc. of

⁶Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 556.

0.2 N NaOH and titrated against phenolphthalein. Calculated for 2 equivalents, 2.32 cc. Found, 2.15 cc.

Ketodilactone, $C_{23}H_{28}O_6$.—A solution of 1 gm. of pseudostrophanthidin in 5 cc. of acetic acid was treated with 2 cc. of chromic acid solution which was prepared by mixing 400 gm. of water, 80 gm. of sulfuric acid, and 53 gm. of chromium trioxide.⁷ The reaction was kept under control by cooling and after completion the mixture was diluted with water. Lustrous leaflets separated on rubbing. The substance was most conveniently recrystallized by dissolving in a necessarily large volume of hot alcohol or acetone, and concentrating to crystallization. It formed small rhombic and wedge-shaped prisms which melted with effervescence at 285° after preliminary softening, and were insoluble in cold alkali. The solution in sulfuric acid gradually developed an amber color.

$$[\alpha]_D^{28} = + 93^\circ (c = 1.01 \text{ in pyridine}).$$

$C_{23}H_{28}O_6$. Calculated.	C 68.97, H 7.05.
Found.	“ 69.03, “ 7.09.

The presence of two lactone groups was shown as follows. 0.1226 gm. of substance was refluxed for 5 hours with 15 cc. of 0.2 N NaOH and titrated against phenolphthalein. Calculated for 2 equivalents, 3.06 cc. Found, 3.00 cc. The identical substance was obtained by replacing in the above oxidation experiment pseudostrophanthidin by the dilactone, $C_{23}H_{30}O_6$. The resulting substance possessed the same properties and melting point as the above oxidation product and a mixed melting point showed no depression.

$$[\alpha]_D^{28} = + 97^\circ (c = 1.01 \text{ in pyridine}).$$

Found.	C 68.73, H 6.92.
--------	------------------

Oxime of the Ketodilactone.—The above oxidation product was refluxed for 2 hours in 20 parts of alcohol with hydroxylamine hydrochloride and sodium acetate. During the reaction the oxime separated incompletely. After collecting it was washed free of salt with water. The mother liquor yielded additional amounts when concentrated. It was recrystallized by dissolving

⁷ Kiliani, H., *Ber. chem. Ges.*, 1913, xlv, 676.

in a necessarily large volume of hot alcohol and by subsequent concentration to crystallization. The oxime formed a sandy deposit of minute rhombs and short, stout prisms which melted with effervescence above 285° .

$C_{23}H_{29}O_6N$. Calculated. C 66.46, H 7.04.
Found. " 66.51, " 6.90.

Oxidation of Dianhydrostrophanthidin.

Dilactone, $C_{23}H_{26}O_4$.—10 gm. of dianhydrostrophanthidin were dissolved in 200 cc. of acetic acid. With cooling the mixture was treated at once with 35 cc. of a Kiliani chromic acid solution.⁷ The mixture was warmed and there was appreciable evolution. After 10 minutes the solution was poured into water. The resulting copious voluminous precipitate rapidly crystallized. After collecting the precipitate with water it was digested with dilute ammonia in order to dissolve small amounts of acid by-products. The ammoniacal extract yielded very small amounts of an acid which will be described below. The neutral precipitate after collecting was dissolved in a necessarily large volume of hot dry acetone and the solution was concentrated to smaller bulk. The dilactone crystallized as lustrous, flat needles and six-sided plates which melted at $253\text{--}254^{\circ}$ after preliminary softening. By working up the mother liquor the yield obtained was 4.2 gm. The substance is readily soluble in chloroform and hot acetic acid, appreciably soluble in acetone, and but sparingly so in alcohol. In sulfuric acid it gives at first a yellowish brown solution which deepens to a deep brown-red and on standing to a deep purple. In dilute pyridine solution it reduces Tollens' reagent due to the unsaturated lactone ring which has resisted the oxidation.

$[\alpha]_D^{25} = -178^{\circ}$ ($c = 1.000$ in chloroform).
 $C_{23}H_{26}O_4$. Calculated, C 75.37, H 7.16.
Found. " 75.49, " 6.94.

Both of the lactone groups were readily saponified and titrated as follows: 0.1013 gm. of substance was refluxed for 2 hours in a mixture of 20 cc. of alcohol and 20 cc. of 0.1 N NaOH and titrated back against phenolphthalein. Calculated for 2 equivalents, 54 cc. Found, 5.7 cc.

With Grignard reagent the substance still shows an active hydrogen atom which is probably associated with the α -carbon atom adjoining the carbonyl and the β -unsaturated carbon atom of the Δ β - γ -crotonic lactone group.

0.0467 gm. substance: 2.72 cc. CH_4 (0° , 760 mm.) or 0.94 mol for mol wt. 366.

Lactone Acid, $\text{C}_{23}\text{H}_{28}\text{O}_5$.—The above described ammoniacal extract of the crude oxidation product when acidified with hydrochloric acid gave a very small amount of amorphous precipitate. The combined material from a number of experiments was boiled up with acetone and the filtrate concentrated to small volume. Rectangular platelets separated which melted with effervescence at 268° .

$$[\alpha]_D^{25} = -100^\circ \quad (c = 1.025 \text{ in } 95 \text{ per cent alcohol}).$$

$\text{C}_{23}\text{H}_{28}\text{O}_5$.	Calculated.	C 71.84, H 7.35.
	Found.	" 71.60, " 7.37.

This acid was also obtained in very small amount by extracting the dilute acetic acid filtrate from the crude oxidation product with chloroform. When shaken out with dilute ammonia the monobasic lactone acid was obtained after acidification. The same acid was obtained as follows when dianhydrostrophanthidin was oxidized in acetone solution with permanganate and no evidence of the formation of the dilactone was obtained. 5 gm. of dianhydrostrophanthidin were dissolved in 300 cc. of hot dry acetone. The solution was cooled to room temperature and then shaken with 3 gm. of potassium permanganate until decolorized a process which took about 15 minutes. The collected precipitate was extracted with water and the filtrate was acidified with acetic acid. The very small amount of crude acid crystallized from dilute acetone as six-sided leaflets which contained 1 mo of water and melted with effervescence at 272 – 274° . It showed no depression when mixed with the lactone acid obtained above as a by-product in the chromic acid oxidation.

$$[\alpha]_D^{25} = -102^\circ \quad (c = 1.025 \text{ in } 95 \text{ per cent alcohol}).$$

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$\text{C}_{23}\text{H}_{28}\text{O}_5 \cdot \text{H}_2\text{O}$.	Calculated.	H_2O 4.47.
	Found.	" 4.13.

Anhydrous Substance.

$C_{23}H_{28}O_5$.	Calculated.	C 71.84, H 7.35.
	Found.	" 71.16, " 7.33.

0.1076 gm. of anhydrous substance when titrated directly against phenolphthalein required 2.9 cc. of 0.1 N NaOH. After the addition of an excess of alkali with subsequent boiling for 4 hours an additional 3.1 cc. were consumed. Calculated for equivalent, 2.8 cc. When this saponification mixture was acidified with acetic acid and the resulting acid was recrystallized from 50 per cent acetic acid it formed fine needles which melted with effervescence at 242–243° and showed no depression when mixed with the dibasic acid which was obtained from the dilactone as follows.

Dibasic Acid, $C_{23}H_{30}O_6$.—The dilactone was saponified as previously described. After neutralization the alcohol was removed under diminished pressure and the mixture was then acidified. The partly crystalline precipitate after collection was recrystallized from 50 per cent acetic acid. It formed needles which melted with effervescence at 249–251° although frequently melting point of 242–243° was observed. The method of recrystallization employed shows the slight tendency to lactonize possessed by the substance. 0.1052 gm. of substance when titrated against phenolphthalein required 5.00 cc. of 0.1 N NaOH. Calculated, 5.24 cc.

$C_{23}H_{30}O_6$.	Calculated.	C 68.61, H 7.52.
	Found.	" 69.04, " 7.43.

Tetrahydrodilactone, $C_{23}H_{30}O_4$.—4 gm. of dilactone were dissolved in about 100 cc. of pure glacial acetic acid and reduced with 0.8 gm. of palladium black and hydrogen. The first mol was absorbed within the first 2 hours and after 24 hours the second mol of hydrogen was used up. The solution when poured to water yielded the crystalline hydrogenation product. When recrystallized from alcohol 2.5 gm. of delicate needles were obtained which melted at 275–277° with slight preliminary sintering. The mother liquor on concentration yielded lower melting material which was not further investigated and which possibly consisted of stereoisomers. The presence of the un-

saturated lactone group was shown by the fact that in dilute pyridine solution it slowly reduced Tollens' reagent. The solubility of the substance decreases in the order chloroform, acetone, alcohol, and benzene.

In sulfuric acid it gives a yellow color.

$$[\alpha]_D^{25} = + 3.0^\circ \text{ (} c = 1.000 \text{ in chloroform).}$$

$C_{23}H_{30}O_4$.	Calculated.	C 74.54,	H 8.17.
	Found.	" 74.37,	" 8.05.

The marked difference in stability of the two lactone groups towards alkali was demonstrated by the following saponification experiments. 0.1109 gm. of substance was refluxed for 2 hours in a mixture of 15 cc. of alcohol and 15 cc. of 0.1 N NaOH and titrated against phenolphthalein. Found, 3.07 cc. Calculated for 1 equivalent, 3.00 cc.

When the saponification mixture was acidified to Congo red a partly crystalline precipitate formed. After collecting with water it was suspended in 50 per cent alcohol and dissolved by addition of ammonia. There remained a small amount of apparently neutral crystalline residue, formed probably by relaxation. The filtrate on acidification yielded microscopic bundles of lens-shaped leaflets of the lactone acid which sintered above 215° and slowly melted at $225\text{--}230^\circ$.

Both lactone groups, however, were saponified under the following conditions. 0.5263 gm. of substance was refluxed for 5 hours in a mixture of 10 cc. of N NaOH and 5 cc. of alcohol. Found, 2.68 cc. Calculated for 2 equivalents, 2.84 cc. When this mixture was acidified a voluminous amorphous precipitate formed which was collected with water. The alcoholic solution was carefully treated with water to incipient turbidity and globular masses slowly separated which were collected with dilute alcohol. The dibasic acid of questionable purity melted at 215° but the amount available made its further study unprofitable. 0.0516 gm. of this substance required 2.10 cc. of 0.1 N NaOH for neutralization. Calculated for 2 equivalents, 2.54 cc.

Hexahydrodilactone, $C_{23}H_{32}O_4$.—After the absorption of the first 2 mols of hydrogen it was found in a number of experiments that further absorption did not occur. This recalls the difficulties experienced with strophanthidin itself. However, when the

lactone was carefully purified and a palladium black was prepared under very careful conditions it was found possible to carry the absorption into the 3 mol stage which, however, required several days for completion. When the reaction mixture was poured into water the hydrogenation product crystallized. After repeated recrystallization from alcohol it formed needles which after slight preliminary sintering melted at 265–267° to a turbid liquid which cleared a few degrees higher. It is easily soluble in chloroform and but sparingly so in the other usual solvents.

The solution in sulfuric acid remains practically colorless. In dilute pyridine solution it does not reduce Tollens' reagent.

$$[\alpha]_D^{25} = + 14^{\circ} (c = 1.015 \text{ in chloroform}).$$

$C_{23}H_{32}O_4$. Calculated. C 74.14, H 8.67.

Found. " 74.38, " 8.79.

Boiling for 2 hours with 0.1 N NaOH resulted in saponification of only one lactone group. 0.1002 gm. of substance was boiled in a mixture of 20 cc. of alcohol and 20 cc. of 0.1 N NaOH. Found, 0.74 cc. Calculated for 1 equivalent, 2.69 cc.

When the saponification mixture was reacidified to Congo red needles of the original dilactone quickly separated as shown by melting point and other properties.

SUBSTITUTION BY HALOGEN OF THE HYDROXYL IN SECONDARY ALCOHOLS.*

BY P. A. LEVENE AND L. A. MIKESKA.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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It is well recognized that the outcome of substitution on the symmetric carbon atom of an optically active substance is determined by the structure and by the polarity of the groups attached to the asymmetric carbon atom, on the one hand, and by the nature of the reacting substance, on the other.

In connection with secondary alcohols, a systematic study of the influences of the groups attached to the asymmetric carbon atom on the outcome of substitution is still missing. Pickard and Kenyon¹ have investigated the behavior of secondary normal alcohols. They observed that halogenation with hydrogen halides brought about a change in the direction of the rotation. McKenzie and Clough have observed that the action of thionyl chloride on methylphenyl carbinol is not accompanied with a change of direction of rotation. More recently Levene and Mikeska² and McKenzie and Tudhope³ have found that the action of thionyl chloride on methylhexyl carbinol caused a change in the direction of rotation.

Thus, up to date, only two alcohols have been tested in regard to differences in the effect of the action of different halogenating agents. In one of the alcohols besides the methyl, a normal hexyl group is attached to the asymmetric carbon atom; in the other, a phenyl group. In the former, there was observed no difference in the effect of the two agents. In the latter, each of the reagents

* This is the fifth paper of the series on Walden inversion.

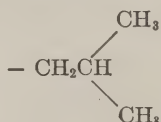
¹ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 45; *Ber. chem. Ges.*, 1912, xlv, 1592.

² Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lix, 473.

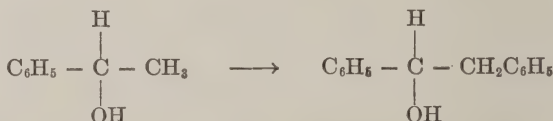
³ McKenzie, A., and Tudhope, T. M. A., *J. Biol. Chem.*, 1924, lxii, 551.

produced a chloride, one enantiomorphous to the other. Hence, in one of two chlorides a Walden inversion took place. The question which now needs an answer is in which of the two the inversion occurred.

We now undertook the study of secondary alcohols in which the groups attached to the asymmetric carbon atom had a different character from the two described above. In one, isobutylmethyl carbinol, the second radicle attached to the asymmetric carbon atom contained a secondary carbon atom



In the other, benzylphenyl carbinol, the two radicles attached to the asymmetric carbon atom contained a benzene ring. It differed from methylphenyl carbinol only in that 1 hydrogen atom in the methyl group of the latter was substituted by a phenyl group.



Besides, an additional representative of the normal secondary alcohol series, ethylmethyl carbinol was tested in respect to its behavior towards thionyl chloride and other halogenating agents.

In each of the three alcohols halogenation was accomplished in two ways. In two, by means of hydrogen iodide and thionyl chloride, in the third by means of phosphorus pentachloride and of thionyl chloride. The action of hydrogen chloride with regard to the resulting direction of rotation is the same as that of hydrogen iodide.

Some interesting peculiarities in the behavior of individual alcohols came to light in the course of this work. In the two alcohols previously tested the chlorination with thionyl chloride proceeded without difficulty by carefully adding the alcohol to the thionyl chloride. The reaction proceeded equally readily in the case of benzylphenyl carbinol but, on the contrary, in the case

ethylmethyl carbinol and of isobutylmethyl carbinol, thionyl chloride, under these conditions, failed to react. Chlorination was brought about only by the method of Darzeus in pyridine solution. Besides, special conditions of temperature and duration of reaction had to be observed in order to avoid racemization. From these observations it seems suggestive that substitutions at the asymmetric carbon atom which proceed readily are accompanied with less racemization than those which take place with difficulty (slowly).

Resolution of Isobutylmethyl Carbinol.—The alcohol used in this experiment was prepared by Grignard's method from isovaleraldehyde and methyl iodide. To resolve the alcohol the general method of Pickard and Kenyon⁴ was employed. The alcohol was converted into a half ester of phthalic acid by heating the molecular proportions of the alcohol and phthalic anhydride at 115°C. for 16 hours on the steam bath under a return condenser. The phthalate was converted into the brucine salt by treating it with the equivalent of brucine in acetone solution. On cooling, the brucinate precipitated. Much difficulty was experienced in separating the dextro from the levo component. If the crystallization was allowed to proceed too long, both forms came down. If interrupted in time, the dextro component was obtained. After many recrystallizations the brucinate was decomposed with hydrochloric acid and the phthalate was isolated by extraction with chloroform. After drying over sodium sulfate, the chloroform was removed. The residue showed a rotation of $[\alpha]_D^{20} = + 51.80^\circ$.

Isobutylmethyl Carbinol.—55 gm. of the above phthalate were steam-distilled with $2\frac{1}{2}$ molecules (25 gm.) of sodium hydroxide. The alcohol when dried weighed 22 gm. and showed a rotation of

$$[\alpha]_D^{20} = \frac{+ 0.98^\circ \times 100}{1 \times 4.3821} = + 22.38^\circ$$

Isobutylmethyl Iodide.—22 gm. of the carbinol obtained in the above experiment were slowly distilled with 80 gm. of hydriodic acid (b.p. = 126°). The iodide obtained was separated by means of a separatory funnel and distilled once more with 80 gm. of hydriodic acid. It was again separated from the aqueous layer, taken out first with a little dilute sodium hydroxide, then with

⁴ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1907, xci, 2058.

water, and finally dried over sodium sulfate. The iodide obtained weighed 22 gm. and showed a rotation of

$$[\alpha]_D^{20} = \frac{-1.24^\circ \times 100}{1 \times 6.855} = -18.08^\circ$$

2-Chloroisohehexane.—10 gm. of isobutylmethyl carbinol, the rotation of which was $[\alpha]_D^{20} = -7.63^\circ$, were mixed with 7.2 gm. of pyridine and then treated with thionyl chloride with just sufficient cooling to keep the temperature below 50°C . The mixture was then allowed to stand at 50° for 3 hours. It was then poured into water, extracted with ether, washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was removed and the residue fractionated:

FI.....b.p. up to 115°C . Weight = 4 gm.

$$[\alpha]_D^{20} = \frac{+2.74^\circ \times 100}{1 \times 21.4} = +12.80^\circ$$

This was combined with the corresponding fraction obtained in another experiment, and the two were redistilled.

FI'.....b.p. up to 108°C .

FII'.....“ $108 - 114^\circ$.

For FII'.

$$[\alpha]_D^{20} = \frac{+3.68^\circ \times 100}{1 \times 18.025} = +20.41^\circ$$

0.1108 gm. substance: 0.1080 gm. AgCl.

$\text{C}_6\text{H}_{13}\text{Cl}$. Calculated. Cl 29.42.

Found. “ 24.11.

Resolution of Benzylphenyl Carbinol.—150 gm. of benzylphenyl carbinol were heated with 118 gm. of phthalic anhydride at 120° for 16 hours. The reaction mixture was then treated with two equivalents of sodium carbonate in aqueous solution and allowed to stand at room temperature for $1\frac{1}{2}$ hours to decompose the unchanged phthalic anhydride. The unchanged carbinol was then extracted with ether and the residue acidified, whereupon the phthalate separated as an oil. It was extracted with chloroform, dried over sodium sulfate to precipitate the phthalic acid, and finally filtered from the phthalic acid and sodium sulfate. Th

chloroform was removed under reduced pressure. The residue crystallized on standing. 220 gm. of the crude phthalate were obtained. It was dissolved in ether and reprecipitated in fine crystalline form with petrolic ether. The phthalate was then dissolved in acetone, treated with a little less than one equivalent of pure quinine, then more quinine was added until the solution showed a slightly alkaline reaction. On cooling, the quinine salt crystallized out. The solution was filtered and the salt extracted with hot acetone to dissolve as much as possible of the levo component. It was then recrystallized several times from 95 per cent alcohol. A fraction was finally obtained which on decomposition gave a phthalate with a rotation of

$$[\alpha]_D^{20} = \frac{+ 1.75^\circ \times 100}{1 \times 6.978} = + 25.08^\circ$$

The phthalate melted at 123°C.

The levo salt could be obtained from the recrystallization mother liquors which, when decomposed, gave a phthalate with a rotation of over $[\alpha]_D^{20} = -20^\circ$.

d-Benzylphenyl Carbinol.—100 gm. of the half ester of *d*-benzylphenyl carbinol and phthalic acid ($[\alpha]_D^{20} = + 25.08^\circ$) were treated with 40 gm. of sodium hydroxide in aqueous solution. The mixture was heated to boiling for about 10 minutes. It was then cooled and the carbinol, which separated as an oil and solidified on cooling, was extracted with ether. The extract was washed with water and dried over sodium sulfate. The solution was concentrated to a small volume and then treated with petrolic ether. The carbinol crystallized on standing. The first crop of crystals weighed 35 gm. 8 more gm. were obtained from the filtrates. Some of the first fraction was dissolved in ether and reprecipitated with petrolic ether. In this state of purity the substance melted at 64°C. and showed a rotation of

$$[\alpha]_D^{20} = \frac{+ 1.68^\circ \times 100}{1 \times 9.060} = + 18.54^\circ$$

d-Benzylphenyl Chloromethane (by Means of Thionyl Chloride).—10 gm. of *l*-benzylphenyl carbinol

$$[\alpha]_D^{20} = \frac{- 0.92^\circ \times 100}{1 \times 9.837} = - 9.35^\circ$$

were added in small amounts to 5 mols of thionyl chloride with cooling. The reaction mixture was then heated under a return condenser on a steam bath for 20 minutes. The excess of thionyl chloride was then removed under reduced pressure at 50–60°C. The residue was dissolved in ether, washed first with water, then with dilute sodium hydroxide, and finally with water again. The ether was removed under reduced pressure of 50°C. If higher temperature were used, the chloride decomposed into stilbene and hydrochloric acid. On cooling below zero the chloride solidified. Its exact melting point, however, was not determined. Without any further treatment the chloride was found to be analytically pure, as is evidenced from the analysis below.

0.1550 gm. substance: 0.1028 gm. AgCl.

C ₁₄ H ₁₃ Cl. Calculated.	Cl 16.38.
Found.	" 16.40.

It had an optical rotation of

$$[\alpha]_D^{20} = \frac{+0.96^\circ \times 100}{1 \times 13.036} = +7.36^\circ$$

l-Benzylphenyl Chloromethane (by Means of Phosphorus Pentachloride).—10 gm. of *d*-benzylphenyl carbinol having a rotation of

$$[\alpha]_D^{20} = \frac{+1.11^\circ \times 100}{1 \times 6.631} = +16.73^\circ$$

were dissolved in 20 cc. of chloroform. This solution was added slowly with cooling to a suspension of 14 gm. of phosphorus pentachloride in 30 cc. of dry chloroform. The mixture was allowed to stand for 1 hour at room temperature. The chloroform and phosphorus oxychloride were then removed under reduced pressure at 45°C. The residue was poured into water and shaken for 1 hour at room temperature to decompose any unchanged oxychloride. The oil was then extracted with ether, washed with dilute sodium hydroxide, then with water, and was finally dried over anhydrous sodium sulfate. When the ether was removed the residue weighed 7 gm. and showed an optical rotation of

$$[\alpha]_D^{20} = \frac{-0.36^\circ \times 100}{1 \times 16.878} = -2.13^\circ$$

CONCLUSIONS.

1. Isobutylmethyl carbinol and benzylphenyl carbinols have been resolved into their active components.
2. Ethylmethyl, isobutylmethyl, and benzylphenyl carbinols have been halogenated by means of reagents of different types. In each of these three alcohols halogenation was always accompanied with a change in the direction of rotation.
3. Ethylmethyl and isobutylmethyl carbinols are chlorinated by thionyl chloride only under definite conditions and their racemization under these conditions is not easily avoided.

ON THE OXIDATION OF SECONDARY MERCAPTANS INTO CORRESPONDING SULFONIC ACIDS.*

BY P. A. LEVENE AND L. A. MIKESKA.

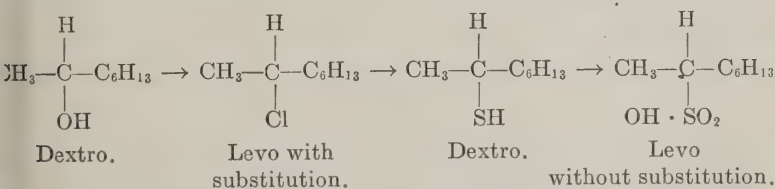
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 30, 1925.)

It was stated in previous publications¹ of this series that the aim of the work was to establish configurational relationships between amino, hydroxy, and halogen derivatives. In the fourth paper of this series, some tentative conclusions were drawn regarding the configurational relations between α -amino and α -hydroxy acids which were based on the observations made on the optical behavior of α -mercapto and α -sulfonic acids.

In the present communication, it will be shown that on the basis of observations on mercapto and sulfonic derivatives of secondary alcohols, certain deductions are permissible concerning the configurational relationships of secondary alcohols and corresponding halides.

In the first communication of this series the following relationships were shown to exist.



In a later communication it was shown that identical relationships existed in the analogous derivatives of ethylmethyl carbinol. In the present communication it will be shown that identical relationships exist between the analogous derivative of isobutyl-

* This is the sixth paper of the series on Walden inversion.

¹Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lix, 473; lx, 685; 1925, lxiii, '85.

methyl carbinol and of benzylphenyl carbinol. The oxidation of the mercapto into the sulfonic derivatives of all these alcohols is accompanied with a change of direction of rotation. Since the differences in the polarity between the OH group and Cl is qualitatively similar to those between the $-SH$ and $-SO_2OH$ groups, it may be concluded that the same configurational relationships exist between the alcohols and halides as between the mercapto and the sulfonic acid derivatives. Hence, it may be concluded that the series of alcohols thus far investigated by us are configurationally related to the halides which rotate in the opposite direction and are enantiomorphously related to those which rotate in the same direction. Attention will be directed next to three other types of alcohols; namely, to methylphenyl carbinol and its homologues, to isopropylphenyl carbinol and its homologues, and, finally, to those in which the aromatic radicles are attached directly to the asymmetric carbon atom.

The case of methylphenyl carbinol presents particular interest in view of the fact that the substitution by halogen of its hydroxyl is accompanied under some conditions with Walden inversion. A decision as to when the inversion occurs will be reached after the mercapto and the sulfonic derivatives of the alcohol have been prepared.

It may be mentioned here that 2-chloroisohehexane, on conversion into the mercapto derivative, is more readily racemized than any of the other halides thus far employed by us.

2-Mercaptoisohehexane.—28 gm. of *l*-2-iodoisohehexane, with an optical activity of

$$[\alpha]_D^{20} = \frac{-1.24^\circ \times 100}{1 \times 6.855} = -18.08^\circ$$

were treated with two equivalents of alcoholic potassium hydrogen sulfide and heated under a return condenser at 60–65°C. for 5 hours. The mixture was then poured into water, whereupon an oil separated on the surface of the aqueous layer. This was separated, washed with a little water, and dried over sodium sulfate. The mercaptan obtained weighed 22 gm. and showed a rotation of

$$[\alpha]_D^{20} = \frac{+0.48^\circ \times 100}{1 \times 8.042} = +5.97^\circ$$

That the above reaction was accompanied with considerable racemization is evident from the fact that in another experiment, carried out under similar conditions, the rotation of the original iodide was

$$[\alpha]_D^{20} = \frac{-1.13^\circ \times 100}{1 \times 6.080} = -18.58^\circ$$

from which a mercaptan was obtained with a rotation of

$$[\alpha]_D^{20} = \frac{+0.64^\circ \times 100}{1 \times 2.921} = +21.21^\circ$$

2-Isohexane Sulfonic Acid.—5 gm. of the mercaptan obtained in the experiment described above were oxidized by heating under return condenser for $1\frac{1}{2}$ hours with 8 cc. of concentrated nitric acid and 2 cc. of water. The substance was then transferred to an evaporating dish and concentrated on the steam bath almost to dryness. A little water was added and the mixture again evaporated almost to dryness. This was repeated until all the nitric acid was removed. The residue was then taken up with water and treated with an excess of barium carbonate. The solution was filtered hot. On concentration, the barium salt precipitated. It was recrystallized from a little water and analyzed.

0.0956 gm. substance: 0.0472 gm. BaSO_4 (for S).

0.1912 " " : 0.1850 " " (" Ba).

$\text{C}_{12}\text{H}_{26}\text{O}_6\text{S}_2\text{Ba}$. Calculated. S 13.70, Ba 29.37.

Found. " 13.29, " 29.05.

To determine the rotation of the salt 1.0632 gm. were dissolved in water and the volume was made up to 10 cc. Rotation was determined in 1 dm. tube.

$$[\alpha]_D^{20} = \frac{-0.52^\circ \times 100}{1 \times 10.632} = -4.89^\circ. \quad [M]_D^{20} = -11.68^\circ.$$

1 cc. of hydrochloric acid was then added, making up the volume to 11 cc. For free acid

$$[\alpha]_D^{20} = \frac{-0.46^\circ \times 100}{1 \times 6.885} = -6.68^\circ. \quad [M]_D = -11.15^\circ.$$

d-Benzylphenyl Mercaptomethane.—14 gm. of *l*-benzylphenyl chloromethane.

$$[\alpha]_D^{20} = \frac{-1.28^\circ \times 100}{1 \times 11.998} = -10.66^\circ$$

were treated with 3 mols of alcoholic potassium hydrogen sulfide solution. The mixture was allowed to stand at the room temperature for $1\frac{1}{2}$ hours which was followed by heating on the steam bath for 2 hours. In order to isolate the mercaptan, the reaction mixture was poured into water and the mercaptan extracted with ether. It was washed with water and dried over sodium sulfate. The ether was removed under reduced pressure at 50°C . At a higher temperature, the mercaptan slowly decomposed to stilbene and hydrogen sulfide. Its rotation was found to be

$$[\alpha]_D^{20} = \frac{+0.58^\circ \times 100}{1 \times 6.774} = +8.56^\circ$$

l-1,2-Diphenylethyl Sulfonic Acid.—3 gm. of benzylphenyl mercaptomethane were dissolved in a mixture of acetone and water. Acetone solution of potassium permanganate was then added at room temperature until the permanganate was no longer being consumed. The excess of permanganate was then removed by adding a little more mercaptan. The manganese dioxide was then filtered off and the filtrate concentrated under reduced pressure. Two immiscible substances, consisting of an oil and the aqueous layer, were obtained. The oil was extracted with ether, washed with water, and dried over sodium sulfate. It showed positive rotation, but was not further investigated.

The aqueous layer was concentrated under reduced pressure. A semicrystalline salt was obtained. This was dried, analyzed, and its optical rotation determined.

$$\text{For the salt } [\alpha]_D^{20} = \frac{-2.06^\circ \times 100}{1 \times 3.817} = -53.96^\circ. \quad [M]_D = -159.5^\circ.$$

$$\text{For the acid } [\alpha]_D^{20} = \frac{-1.70^\circ \times 100}{2 \times 2.335} = -36.40^\circ. \quad [M]_D = -95.36^\circ.$$

0.1338 gm. substance: 0.1082 gm. BaSO_4 (for S).

0.0941 " " : 0.0259 " K_2SO_4 (" K).

$\text{C}_{14}\text{H}_{13}\text{SO}_3\text{K}$. Calculated. S 10.67, K 13.02.
Found. " 11.11, " 12.35.

THE DISSOCIATION CONSTANTS OF PLANT NUCLEOTIDES AND NUCLEOSIDES AND THEIR RELATION TO NUCLEIC ACID STRUCTURE.

BY P. A. LEVENE AND H. S. SIMMS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I. INTRODUCTION AND THEORY.

Several theories have been advanced regarding the mode of union between the nucleotides in the nucleic acids. One possible mode of union is an "ether" linkage from one carbohydrate group to another. Another possible linkage is from the phosphoric acid group of one nucleotide to the carbohydrate group of the next, thus leading to a complex "ester."

The theory of the latter linkage has been supported in the case of animal (thymus) nucleic acid by direct chemical proof. This "ester" structure proposed by Levene has not been disputed and additional evidence in its favor will be presented in this paper.

The structure of plant (yeast) nucleic acid has, on the other hand, been subject to considerable controversy. According to Jones' original theory, the four nucleotides which compose this molecule are linked through the sugar groups. His later structure called for one such (*ether*) linkage and two *ester* linkages. According to Levene, however, all three linkages are *ester* linkages in both plant and animal nucleic acid.

As a possible means for determining the correct structure, we may observe that, in addition to the acidic and basic groups on the "bases,"¹ there should be *eight* ionizable hydrogen atoms on the phosphoric groups, according to Jones' original structure; *six*, according to his later structure; and *five*, according to the structure of Levene.

¹ We retain the old (but misleading) term "base" to refer to a purine or pyrimidine derivative, even though some are acids or ampholytes.

Unfortunately, the determination of the total number of ionizable groups by direct titration (either colorimetric or electrometric) is prevented by the insolubility in acid solution, the possibility of hydrolysis, and the difficulty of working in strongly acid and, particularly, in strongly alkaline solutions. (As may be seen from the results in this paper, even the nucleotides give uncertain results in alkaline solutions.)

The problem can be solved, therefore, only by calculating the number of groups which should dissociate *within a certain pH range*, according to each of the proposed structures; and then determining the actual values experimentally. For this purpose, we have determined the dissociation constants of the nucleosides and the nucleotides, in order to be able to predict the constants for nucleic acid. These experimental values are presented in this paper.

II. RESULTS.

Table I gives the pK' value² of each ionizable group of each plant nucleoside and nucleotide as obtained by electrometric titration. Three of the four constants of guanylic acid have also been determined by Hammarsten.

Since a nucleoside consists of the (sugar-"base") complex, the only ionizable groups are those of the "base." In the nucleotides ($PO(OH)_2 \cdot O$ -sugar-"base") we have, in addition, two ionizable H atoms on the phosphoric group.

The consistency of each type of group is demonstrated in Fig. 1 in which the degree of dissociation is plotted against pH. The dotted curves refer to nucleosides and the solid lines to nucleotides. The curves for the phosphoric groups (lighter lines) show that these pK' values correspond approximately to (but are a little lower than) the first and second pK values of phosphoric acid (*i.e.*, 2.0 and 6.8). The amino groups are also essentially

² The term pK' (which equals $-\log K'$) is used to refer to the constant not corrected for activity. Thus for an acid group: $K' = K \frac{f_i}{f_u}$ where f_u is the activity coefficient of a molecule in which the group in question is unionized, and f_i is the activity of the molecule when that group is ionized. All the constants given in this article are uncorrected. The corrected constants for acid groups would be about 0.1 pH unit higher; and for amino groups, about 0.1 pH unit lower.

like and have constants in the same order of magnitude as aniline ($pK = 4.7$), except in the guanine derivatives. The constants of the first hydroxyl groups (except in the cytosine derivatives) are essentially the same as phenol ($pK = 10$). The difference between the two hydroxyl pK' values in the uracil

TABLE I.
pK' Values of Nucleosides and Nucleotides.

	Nucleosides.	Nucleotides.	See Table No.	See Fig. No.	First H_2PO_4R ionization.	NH_2 group hydrolysis.	Second H_2PO_4R ionization.	First OH group.	Second OH group.
Purine derivatives.	Adenosine.	Adenylic acid.	II	1, A		3.45			
			VIII	1, A	0.89	3.70	6.01		
	Guanosine.	Guanylic acid.	III	1, B		1.6		9.16	
			IX	1, B	0.7	2.3	5.92	9.36	
Pyrimidine derivatives.	Cytidine.	Cytidine phosphoric acid.	IV	1, C		4.22		12.3	
			X	1, C	0.80	4.24	5.97	(13.2)	
	Uridine.	Uridine phosphoric acid.	V	1, D				9.17	12.52
			XI	1, D	1.02		5.88	9.43	(13.9)

In addition, the "base," *uracil*, was found to have pK' values of 9.28 and 13.56, due to its two hydroxyl groups.

Inosine (deaminated adenosine) has a value of 8.72 for its hydroxyl group (compare with the phenolic group of guanosine).

derivatives (*i.e.*, $pK_{a1}' - pK_{a2}'$) corresponds with the molecular dimensions and electrostatic forces.

The pK' values below 1, and particularly those above 12 or 13 are not accurate owing to experimental difficulties. The other values are reasonably accurate (but are not corrected for activity).

There is every reason to believe that the animal nucleosides and nucleotides should have almost identical constants with those from plants.

III. BEARING ON THE STRUCTURE OF NUCLEIC ACID.

If we collect all the pK' values found in the "bases" of the four nucleotides, we obtain the values represented in Fig. 2, A. These represent the pG' values³ of these groups which will be

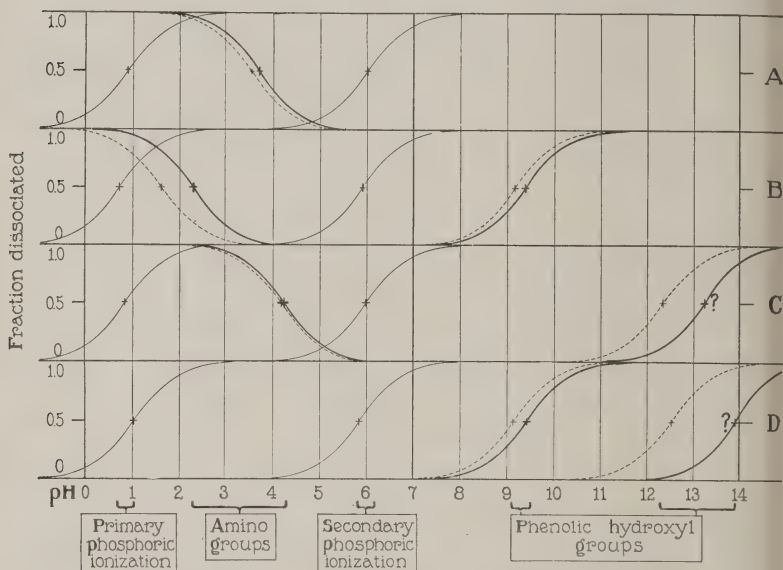


FIG. 1. Dissociation curves of the nucleotides (solid lines) and nucleosides (dotted lines). The lighter lines represent the dissociation of the phosphoric groups. The curves for the amino groups are reversed, thus representing their ionization.

A = adenylic acid and adenosine; B = guanylic acid and guanosine; C = cytidine phosphoric acid and cytidine; and D = uridine phosphoric acid and uridine.

³ The G' constants are those found by treating the titration data as if the solutions contained equivalent quantities of monovalent acids. When the pG' values are two or more pH units apart the G' constants equal the classical K' constants. For any two pG' values which are closer together we have the relation:

$$K_1' = G_1' + G_2' \quad \text{and} \quad (1)$$

$$\frac{1}{K_2'} = \frac{1}{G_1'} + \frac{1}{G_2'} \quad (2)$$

found in nucleic acid according to either of the proposed structures; providing the groups are sufficiently distant in space to eliminate the mutual electrostatic effect. (The actual distances are large enough to make this effect small.)

In Fig. 2, *B* are the phosphoric pG' values to be expected from Levene's theory of either plant or animal nucleic acid. They are spread apart to an extent roughly calculated for the distances between the groups (in space) and the electrostatic forces. Simi-

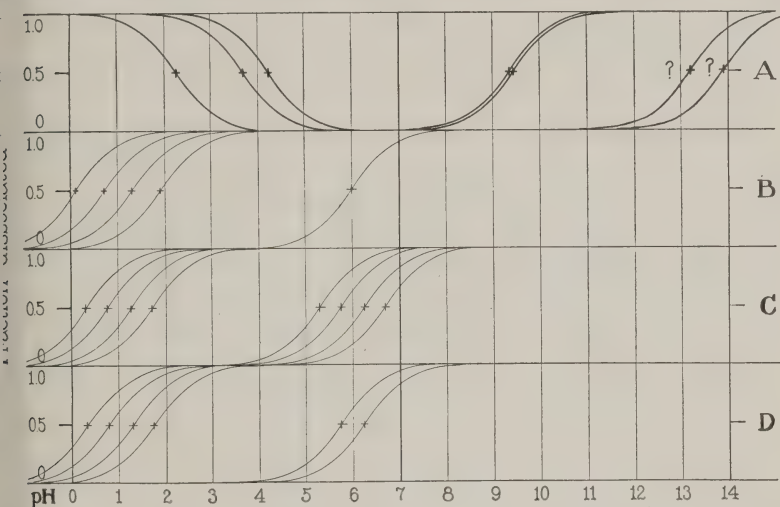


FIG. 2. Predicted approximate dissociation curves for nucleic acid.

A = phenolic and amino groups on the "bases" (according to any structure); *B* = phosphoric groups according to Levene's structure for plant (or animal) nucleic acid; *C* = phosphoric groups according to Jones' original structure for plant nucleic acid; and *D* = phosphoric groups according to Jones' later structure for plant nucleic acid.

larly, Fig. 2, *C* gives the dissociation curves of the phosphoric pG' values according to Jones' original theory, and Fig. 2, *D* for his later theory of plant nucleic acid.

The essential difference between these structures lies in the number of "secondary" phosphoric groups (*i.e.*, the pG' values

When there are three or more groups close together, similar formulas may be used to calculate the K' constants. Since the G' constants are more useful for the present purpose, we need not consider the K' constants. For the nucleosides and nucleotides the K' constants may be considered equal to the corresponding G' constants, but this is not true for nucleic acid.

near pH 6). Any method for determining either the number of groups in that range or for determining the total number of groups below pH 8 (or any higher pH) will distinguish between the structures.

It will be seen from Fig. 2 that the following number of (acid) groups are ionized at the indicated pH values.

pH.....	5.0	8.0	11.0
Levene's structure.....	4	5	7
Jones' original structure.....	4	8	10
“ later “	4	6	8

Thomas and Dox⁴ have analyzed sodium and sodium-ammonium salts of plant (yeast) nucleic acid and have found from 4 to 7 or 8 equivalents of alkali in solutions ranging from acid to alizarin, to alkaline to phenolphthalein. By disregarding the phenolic dissociation of the “bases,” they assumed that this amount of alkali was combined with the phosphoric groups and that Jones' theory was supported. It will be seen from Fig. 2, however, that solutions which are alkaline to phenolphthalein (pH 10 or above) are in the range of dissociation of the phenolic groups, so that the data of Thomas and Dox support the theory of Levene rather than that of Jones. Their solutions containing only 5 or 6 equivalents of alkali were alkaline to phenolphthalein which agrees with Levene's theory⁵ and not with Jones'. Only one of their seventeen solutions definitely disagrees with the predicted values for Levene's structure. That solution (Series I, Preparation *a*) was neutral to phenolphthalein but contained only 4 (instead of 5) equivalents of alkali (Jones' original structure would require 8, and his later structure, 6). The results of Thomas and Dox are summarized in Table II.

⁴ Thomas, A., and Dox, A. W., *Z. physiol. Chem.*, 1925, cxlii, 1.

⁵ Since they give no exact pH values it is hard to interpret the data of Thomas and Dox. A solution containing 5 equivalents of alkali should have a pH between 7 and 8 (according to Levene's structure) and a slight excess of alkali (*e.g.*, 0.2 equivalent) would bring it in the range where phenolphthalein would show pink and might be called “alkaline.” (See Series III, Table II.) Certainly the data do not correspond with either structure of Jones, since these would give solutions distinctly acid to phenolphthalein with 5 (or with 5.2 or even 5.8) equivalents of alkali.

TABLE II.

Data of Thomas and Dox, on the Number of Equivalents of Base Combined with Nucleic Acid at Various Reactions.

Series.....	I			II		III		IV		V	
	Eq.*	Aliz.	Pp.	Eq.	Pp.	Eq.	Pp.	Eq.	Pp.	Eq.	Pp.
Preparation a.	4†	Alk.	Neut.†	7	Alk.	5	Alk.	7	Alk.	8	Alk.
“ b.	4	“	Acid.	4	Acid.	5	“	7	“	8	“
“ c.	4	“	“	4	“	5	“	6+	“	7	“
“ d.	4	Acid.	“	4	“						

* Eq. = number of equivalents of base (sodium plus ammonia); Aliz. = reaction toward alizarin; Pp = reaction toward phenolphthalein; Alk. = alkaline; Neut. = neutral.

† Series I, Preparation a, is the only one which does not correspond with Levene's structure.

We have made complete titration curves of nucleic acid and although our present values on yeast nucleic acid are unsatisfactory, we find the following tentative pG' values for thymus nucleic acid (calculated by a graphical modification of the "buffer value" method of Van Slyke⁶) between pH 3 and 10. The approximate predicted values (see Fig. 2) are given for comparison and also some pK' values from constants obtained by Hammarsten⁷ on thymus nucleic acid.

	pG_6'	pG_7'	pG_8'	pG_9'	pG_{10}'	pG_{11}'	pG_{12}'	pG_{13}'
Predicted for Levene's structure (plant or animal).....	3.7	4.2	(6.0)	9.4	9.5			
Found by Levene and Simms (animal).....	4.0	4.3	6.3	9.5	9.7			
Found by Hammarsten ⁷ (animal)	3.7	4.3	5.2					
Predicted for Jones' original structure (plant).....	3.7	4.2	(5.4)	(5.8)	(6.2)	(6.6)	9.4	9.5
Predicted for Jones' later structure (plant).....	3.7	4.2	(5.8)	(6.2)	9.4	9.5		

The values in the range from pH 4.5 to 9 are given in bold faced type. It is evident that there is *but one* pG in this range; which agrees with the structure of Levene.

⁶ Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.

⁷ Hammarsten, E., *Biochem. Z.*, 1924, cxliv, 383. The formulas used by Hammarsten for calculating the constants are theoretically incorrect

These values for thymus nucleic acid are only tentative and we also intend to obtain better data on yeast nucleic acid. We present the results in this paper on nucleosides and nucleotides as a preliminary communication.

IV. EXPERIMENTAL.

Sufficient material was weighed out in each case (allowing for moisture) to make a mother solution twice the strength indicated and sometimes containing exactly 1, 2, or 3 equivalents of molar strong acid or alkali. From these mother solutions, 5.0 cc. samples were diluted to 10.0 cc. (sometimes 2.5 cc. samples were diluted to 5.0 cc.) in volumetric flasks, after the required amount of tenth molar acid or alkali had been added.⁸ These solutions had the indicated concentrations and, "equivalents of base" $\left(\frac{B-A}{C}\right)$. Their pH values were determined and are given in Tables III to XII.

The constants are calculated by the formulas:

$$\text{and} \quad G'_z = H \frac{\alpha_z}{1 - \alpha_z} \quad (3)$$

$$B' + y = \frac{h + B - A - oh}{C} + y = \alpha_1 + \alpha_2 + \alpha_3 + \text{etc.} = \Sigma \alpha \quad (4)$$

where G'_z is a constant (not corrected for activity) which, in all the nucleosides and nucleotides, is equal to either a dissociation

when applied to nucleic acid, and give values which are only roughly correct. We have not recalculated his data since the experimental error is large and also because it would be more important to know the values of the constants above pH 6 than to have more accurate values of those which he determined.

His formulas make correction for incomplete dissociation of groups with lower pG' values, but not for partial dissociation of groups with higher pG' values. Both corrections are equally necessary.

⁸ It is interesting to observe that in neutralizing some alkaline solution of guanosine with HCl, a clear, transparent, viscous gel was first produced. In the course of a few seconds, small crystals formed which increased in number as the viscosity simultaneously dropped, until a thin watery suspension of finely precipitated material remained. The same cycle took place with another solution containing alcohol but was prolonged over a period of several minutes.

constant (K_a') of an acid group or the hydrolysis constant (K_b') of a basic group. (There are many compounds, however, in which the G' constants cannot be considered equal to the K' constants, but the relation can then be found by equations (1) and (2) in Foot-note 3.)

$$B' = \frac{h+B-A-oh}{C} \text{ is the "corrected equivalents of base."}$$

y = number of groups which ionize as bases (amino groups).

h = hydrogen ion *concentration*, where H is its *activity*.

oh = hydroxyl " " " OH " " "

B = molar concentration of strong base (NaOH).

A = " " " " acid (HCl).

C = " " " substance.

z = any subscript. (The G' constants are numbered in order of their numerical value.)

When the pG' values are so close together that formulas (1) and (2) must be used, α_1, α_2 , etc., do not correspond to any molecular or ionic concentrations. However, in cases such as the nucleosides and nucleotides where the pG' values are far apart, α_2 is the fraction of the substance in that form which predominates at a higher pH, and $(1 - \alpha_2)$ is the fraction in that form which predominates at a lower pH, than pG_z' , whether the group is acidic or basic.

All pH values were determined at 25.0°C. in a water-jacketed bubbling electrode. The pH of tenth normal hydrochloric acid was taken as 1.090 and the liquid junction potential with the saturated KCl bridge was assumed constant. The data are given in Tables III to XII.

TABLE III.
Adenosine. (0.0500 Molar.)

pH	$\frac{B-A}{C}$	B'	α	pG'
4.41	-0.100	-0.100	0.900	3.45
4.06	-0.200	-0.199	0.801	3.46
3.84	-0.300	-0.279	0.721	3.47
3.60	-0.400	-0.394	0.606	3.41
3.30	-0.600	-0.589	0.411	3.46
2.89	-0.800	-0.771	0.229	3.41
2.43	-1.000	-0.917	0.083	3.47
Amino group: $pK_b' =$				3.45

TABLE IV.
Guanosine. (0.0250 Molar.)

pH	$\frac{B-A}{C}$	B'	α_1	pG ₁ '	α_2	pG ₂ '
1.543	-1.800	-0.513	0.487	1.57		
1.14	-3.800	-0.755	0.245	(1.66)		
9.88	0.90	0.895			0.895	(8.95)
9.83	0.84	0.835			0.835	9.13
9.78	0.80	0.795			0.795	9.20
Amino group: $pK_b' =$				1.6		
Hydroxyl group: $pK_a' =$						9.16

TABLE V.
Cytidine (Sulfate). (0.0500 Molar.)

(B' is here calculated as if an equivalent of H_2SO_4 had been added to a solution of cytidine; e.g., $\frac{A}{C} = 1$.)

pH	$\frac{B-A}{C}$	B'	α_1	pG ₁ '	α_2	pG ₂ '
3.33	-0.900	0.890	0.110	4.23		
3.84	-0.700	0.698	0.302	4.21		
4.22	-0.500	0.500	0.500	4.22		
4.58	-0.300	0.300	0.700	4.21		
11.83	0.500	0.300			0.300	12.20
12.05	0.650	0.320			0.320	12.38
12.27	0.900	0.35			0.35	12.54
12.20	1.000	0.54			0.54	12.27
Amino group: $pK_b' =$				4.22		
Hydroxyl group: $pK_a' =$						12.3

TABLE VI.
Uridine. (0.0500 Molar.)

pH	$\frac{B-A}{C}$	B'	α_1	pG_1'	α_2	pG_2'
2.04	-0.200	0	0		0	
8.64	0.200	0.200	0.200	9.24		
8.73	0.300	0.300	0.300	9.10		
9.03	0.400	0.400	0.400	9.16		
9.16	0.500	0.500	0.500	9.17		
9.34	0.600	0.600	0.600	9.17		
9.74	0.800	0.798	0.798	9.17		
11.88	1.40	1.18			0.18	12.55
12.07	1.60	1.26			0.26	12.52
12.19	1.80	1.33			0.33	12.50
12.31	2.00	1.39			0.39	12.51
Hydroxyl groups $\begin{cases} pK_{a1}' = \dots\dots\dots \\ pK_{a2}' = \dots\dots\dots \end{cases}$				9.17		12.52

TABLE VII.
Uracil. (0.0500 Molar.)

(The last three measurements were made in 0.500 molar solutions.)

pH	$\frac{B-A}{C}$	B'	α_1	pG_1'	α_2	pG_2'
8.66	0.200	0.200	0.200	9.26		
9.10	0.400	0.400	0.400	9.28		
9.46	0.600	0.600	0.600	9.28		
9.87	0.800	0.798	0.798	9.28		
12.13	1.400	1.025			0.025	(13.70)
12.39	1.800	1.065			0.065	13.55
12.49	2.000	1.080			0.080	13.55
13.11	1.600	1.23			0.23	13.62
13.22	1.800	1.31			0.31	13.57
13.32	2.000	1.38			0.38	13.53
Hydroxyl groups $\begin{cases} pK_{a1}' = \dots\dots\dots \\ pK_{a2}' = \dots\dots\dots \end{cases}$				9.28		13.56

TABLE VIII.
Inosine. (0.0240 Molar.)

pH	$\frac{B-A}{C} = B' = \alpha$	pG'
9.40	0.833	8.70
8.96	0.625	8.74
8.57	0.417	8.72
8.14	0.208	8.72
Hydroxyl group: $pK_a' = \dots\dots\dots$		8.72

TABLE IX.

Adenylic Acid. (0.0250 Molar.)

(pG_2' was calculated before pG_1' in order that it might be used to obtain the values of α_2 in the fourth column.)

pH	$\frac{B-A}{C}$	B'	α_2 (calculated).	$\frac{\alpha_1}{HB'} - \frac{1}{\alpha_2}$	pG_1'	α_2	pG_2'	α_3	pG_3'
(2.08)	-0.400	-0.03	0.02	0.95	(0.80)				
(1.94)	-0.600	-0.08	0.02	0.90	(0.99)				
1.707	-1.000	-0.12	0.01	0.87	0.89				
1.223	-3.000	-0.31	0.01	0.68	0.89				
3.17	0.200	0.231				0.231	3.69		
3.55	0.400	0.413				0.413	3.70		
3.71	0.500	0.509				0.509	3.69		
3.90	0.600	0.606				0.606	3.71		
4.34	0.800	0.802				0.802	3.74		
5.49	1.200	1.200						0.200	6.06
5.84	1.400	1.400						0.400	6.01
6.00	1.500	1.500						0.500	6.00
6.17	1.600	1.600						0.600	6.00
6.62	1.800	1.800						0.800	6.02
9.34	2.000	2.000							
11.53	2.200	2.000							
First phosphoric ionization: $\text{pK}_{\text{p1}}' = \dots$					0.89				
Amino group: $\text{pK}_b' = \dots$							3.70		
Second phosphoric ionization: $\text{pK}_{\text{p2}}' = \dots$									6.01

TABLE X.

Guanylic Acid. (0.0250 Molar.)

(pG₂' was calculated before pG₁' in order that it might be used to obtain the value of α_2 in the fourth column.)

pH	$\frac{B-A}{C}$	B	α_2 (calculated).	$\frac{\alpha_1}{HB'} - \alpha_2$	pG ₁ '	α_2	pG ₂ '	α_3	pG ₃ '	α_4	pG ₄ '
1.10	-4.00	-0.46	0.06	0.48	(1.13)						
1.21	-3.00	-0.24	0.07	0.69	0.86						
1.39	-2.00	-0.17	0.10	0.73	0.97						
1.61	-1.000	+0.102	0.160	0.940	0.41						
1.78	-0.600	0.145	0.218	0.927	0.67	0.222	2.32				
1.81	-0.500	0.195	0.231	0.964	0.39						
2.08	0	0.373				0.411	2.24				
2.66	0.600	0.695				0.695	2.30				
3.07	0.800	0.838				0.838	2.36				
4.37	1.000	1.002									
5.34	1.200	1.200						0.200	5.94		
5.77	1.400	1.400						0.400	5.94		
6.09	1.600	1.600						0.600	5.92		
6.49	1.800	1.800						0.800	5.89		
7.44	2.000	2.000									
8.79	2.200	2.200								0.200	9.39
9.24	2.400	2.400								0.400	9.38
9.53	2.600	2.598								0.598	9.36
First phosphoric ionization: pK _{p1} ' =					0.7						
Amino group: pK _b ' =							2.3				
Second phosphoric ionization: pK _{p2} ' =									5.92		
Hydroxyl group: pK _a ' =											9.38
"First," "second," and "third" constants of Hammarsten:							2.4		6.1		9.7

TABLE XI.
Cytidine Phosphoric Acid. (0.0259 Molar.)

[illegible]

TABLE XII.

Uridine Phosphoric Acid. (0.0250 Molar.)(The ammonium salt was used for determining pG_1 and pG_2 , but a sample of free acid was used at higher pH values.)

pH	$\frac{B-A}{C}$	B'	α_1	pG_1'	α_2	pG_2'	α_3	pG_3'	α_4	pG_4'
1.405	-1.000	0.76	0.76	(0.91)						
1.482	-0.800	0.68	0.68	(1.16)						
1.523	-0.600	0.74	0.74	1.16						
1.572	-0.400	0.802	0.802	0.97						
1.642	-0.200	0.823	0.823	0.98						
1.726	0	0.843	0.834	1.00						
1.954	0.400	0.900	0.900	1.01						
2.145	0.400	0.922	0.922	1.07						
3.83	1.000	1.006								
5.27	1.200	1.200			0.200	5.87				
5.71	1.400	1.400			0.400	5.89				
6.07	1.600	1.600			0.600	5.90				
6.45	1.800	1.800			0.800	5.85				
9.03	2.280	2.280					0.280	9.44		
9.40	2.480	2.480					0.480	9.43		
9.77	2.680	2.676					0.676	9.44		
0.25	2.880	2.870					0.870	9.42		
1.16	3.08	2.99								
1.91	3.48	3.00								
2.17	3.88	3.00								
2.34	4.28	2.99								
2.83	7.08	3.08							0.08	13.9
(Other readings unsatisfactory.)										
First phosphoric ionization: $pK_{p1}' = \dots$				1.02						
Second phosphoric ionization: $pK_{p2}' = \dots$						5.88				
First hydroxyl group: $pK_{a1}' = \dots$								9.43		
Second hydroxyl group: $pK_{a2}' = \dots$										(13.9)

V. SUMMARY.

The fifteen dissociation constants of the four nucleotides which compose a plant nucleic acid molecule; and also the seven constants of the four corresponding nucleosides have been determined.

The amino groups give values near that of aniline; the hydroxyl groups give values in the order of magnitude of phenol; while the primary and secondary ionizations of the phosphoric groups are close to those of phosphoric acid.

The various structures proposed for nucleic acids should have essentially identical constants except in the range near pH 6. In that range, Levene's structure for plant nucleic acid (and also his structure for animal nucleic acid) requires *one* group; while the structures proposed by Jones for plant nucleic acid call for *two* or *four* groups in the same range.

Data obtained by Thomas and Dox, although interpreted differently by them, support the structure of Levene for plant nucleic acid.

Tentative constants of animal (thymus) nucleic acid obtained by Levene and Simms also substantiate the structure of Levene for this substance.

THE NUMERICAL VALUES OF THE OPTICAL ROTATION OF METHYLATED GLUCONIC ACIDS AND OF THEIR SALTS.

BY P. A. LEVENE AND G. M. MEYER.

From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 6, 1925.)

The observations on the optical rotations of sugar acids and their salts and on the α substituted acids and their salts have led to the general conclusion that the rotations of the free acids have a direction to the left from their metallic salts for all those hydroxy acids in which the allocation of the hydroxyl is the same as that of carbon atom (2) of gulonic acid; that is, the difference between the numerical values of the rotations of the free acids and of their salts has a minus sign. The same is true for 2-aminoheptonic acids and for α -amino acids. The rule is reversed for the acids in which carbon atom (2) has the configuration of mannonic acid. This rule held for all free acids thus far observed, and it was therefore suggested as a basis for differentiation between the members of the α substituted acids of the *l* and of the *d* series.

Observations on other derivatives of α -hydroxy acids are not quite so numerous. They were entirely lacking on the methyl ethers of sugar acids, and yet a knowledge of the optical behavior of these derivatives seemed desirable for the sake of finding an explanation for the differences in the rotations of free acids and of their salts.

From the observations of previous workers it is known that methoxy-*l*-propionic acid (derived from *l*-lactic acid (dextro)) has a molecular rotation for the free acid $[M]_D = -66^\circ$ ($c = 3$ to 4); the Na salt has $[M]_D = -62.8^\circ$; and other salts also show a decreasing levorotation whereas *l*-lactic acid is dextrorotatory and its salts are levorotatory.

In the case of malic acid, the *d* acid has a specific rotation

($c = 10$) of $+2.17^\circ$ and the potassium salt $+6.78^\circ$; on the other hand, *d*-methoxysuccinic acid has a specific rotation of $+33.3^\circ$ ($c = 10.8$) whereas the neutral potassium salt has the specific rotation of $+9.54^\circ$ ($c = 12.16$).

For the sugar acids, to our knowledge, observations are recorded only on methylated mannonic acids; namely, on 2,3,5,6-tetramethyl and 2,3,4,6-tetramethyl mannonic acids.

Two peculiarities were observed on these two derivatives. The 2,3,5,6-tetramethyl mannonic acid was levorotatory whereas the 2,3,4,6-tetramethyl derivative was dextrorotatory. This difference in direction of rotation of two derivatives differing only in that one of the methyl groups in one was in position 4 and in the other, in position 5, seemed rather surprising. Furthermore, the difference between the rotations of the free acids and their sodium salts had a minus sign, as it is in the *d*-gluconic acid series of unsubstituted acids.

With a view of finding an explanation for the differences in the optical behavior of two substances so closely related, it was considered expedient to prepare a series of partially methylated and of completely methylated gluconic acids.

There was still another interest attached to the present investigation. It was pointed out some time ago by one of us that 2,5-anhydrogluconic acid and 2,5-anhydromannonic acid show exactly the same molecular rotations either as salts or as free acids. This peculiarity was explained by the rigidity of the structures of these two acids. However, *a priori*, another factor might have been held responsible for this phenomenon; namely, the absence of replaceable hydrogens on carbon atoms (2) and (5). The behavior of the methylated gluconic acids should furnish the data required for choosing between the two possible explanations. This problem may be dismissed without much discussion, since in all methylated gluconic acids, the molecular rotations of the acids differed from those of their salts. Thus, these observations lent support to the first of the two above mentioned theories.

The details of the optical behavior of the methylated gluconic acids are given in Table I.

From this table it is seen that in the series of methylated gluconic acids the difference in rotations of the free acids and their

salt has a minus sign as is the case in the free gluconic acid. In this respect the methylated gluconic acids differ from mannonic acid.

The table further shows: first, that all methylated acids have a higher dextrorotation than the mother substance; second that two acids containing an equal number of methyl groups may have different molecular rotations when the methyl groups are differently distributed. Thus, 2,3,5- and

TABLE I.

Specific and Molecular Rotation of Methylated Gluconic Acids.

Methylated derivative.	Solution in 1 equivalent NaOH.		The same solution neutralized.	
	$[\alpha]_D$	$[M]_D$	$[\alpha]_D$	$[M]_D$
2,3-Dimethyl.....	+43.7	+97.46	+22.5	+50.17
2,5,6-Trimethyl.....	+24.0	+56.88	-6.35	-15.05
2,3,5-Trimethyl.....	+64.4	+152.62	+19.3	+55.74
2,3,5,6-Tetramethyl.....	+76.4	+191.76	+43.4	+108.93
2,3,4,5,6-Pentamethyl.....	+53.7	+141.30	+22.5	+59.62

TABLE II.

Change in the Numerical Value of Rotation Due to Substitution in Different Positions.*

Carbon atom No.	$[M]_D$
2	+134.88
3	-63.02
4	-50.46
5	+55.16
6	+39.14

* These values are calculated from the molecular rotation in alkaline solution of Table I less that of gluconic acid $[M]_D = 25.60$. It is not implied that these values will hold for other combinations.

2,5,6-trimethyl gluconic acids have different molecular rotations. Particularly striking is the fact that the pentamethylated acid has a lower molecular rotation than some of the partially methylated acids. From these the conclusion immediately follows that whereas the methylation of some of the hydroxyls augments the rotation of the mother substance, the methylation of the others causes a drop in the rotation.

In fact, from the data given in Table I, it is possible to calculate

the effect on the total molecular rotation of the methylation of each one of the carbon atoms separately. These values are given in Table II. Plus sign indicates an enhancing influence minus sign a depressing one.

It is expected to obtain data on the molecular rotation of the methylated derivatives of the other sugar acids in the hope that a more comprehensive explanation of the optical behavior of these substances may be arrived at.

CONCLUSIONS.

1. In all methylated gluconic acids, the difference in the optical rotations of the free acids and of their salts has a minus sign.

2. The influence on the value of the molecular rotation of the methylation varies with the change in position of the methyl groups.

3. Further evidence is furnished to the theory that the peculiarity in the behavior of 2,5-anhydrosugar acids is due to the rigidity of their structures.

EXPERIMENTAL.

2,3-Dimethyl Gluconic Lactone.

Benzylidene Methyl Glucoside.—The method of Irvine¹ was slightly modified: 50 gm. of methyl glucoside, 300 cc. of freshly distilled benzaldehyde, and 50 gm. of anhydrous sodium sulfate contained in a distillation flask were heated in an oil bath at 145°C. for 2 hours and then at 165°C. for 5 hours. The benzaldehyde was removed as far as possible by distillation under reduced pressure (oil bath temperature 125°C.). The contents of the flask were extracted with dry neutral ethyl acetate and filtered from the sodium sulfate. On cooling with ice and salt the benzylidene compound was obtained as a crystalline mass. It was filtered on suction and washed with cold ether. This removed most of the coloring water and left the benzylidene glucoside as a nearly white crystalline powder. The ethyl acetate filtrate was concentrated under reduced pressure and poured into

¹ Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 580.

a large volume of petroleic ether. The precipitate was recrystallized from ethyl acetate. The total yield of crude material from 100 gm. of glucoside was 145 gm. This product was recrystallized from methyl alcohol and it then had a melting point of 160–161° and analyzed as follows:

0.0966 gm. substance: 0.2138 gm. CO_2 and 0.0582 gm. H_2O .

$\text{C}_{14}\text{H}_{18}\text{O}_6$. Calculated. C 59.57, H 6.38.

Found. " 60.38, " 6.74.

2,3-Dimethyl Benzylidene Methyl Glucoside.—This was prepared from the benzylidene glucoside according to the directions of Irvine. It melted at 122–123°C. and had the following composition.

0.1028 gm. substance: 0.2322 gm. CO_2 and 0.0662 gm. H_2O .

$\text{C}_6\text{H}_{22}\text{O}_6$. Calculated. C 61.89, H 7.12.

Found. " 61.57, " 7.20.

2,3-Dimethyl Glucose.—The previous compound was boiled with 10 per cent hydrochloric acid for 30 minutes. The solution was cooled and the benzaldehyde removed in a separatory funnel with ether. The aqueous part was neutralized with barium carbonate and the methylated sugar isolated by the usual procedures. No attempt was made further to purify the product.

2,3-Dimethyl Gluconic Lactone.—35 gm. of the syrup obtained by the hydrolysis of dimethyl benzylidene glucoside were oxidized with bromine at 30–35°C. and constant stirring until it failed to reduce Fehling's solution. The excess bromine was removed under reduced pressure and the hydrobromic acid with silver carbonate according to the usual procedure.

The syrup which remained after removal of the solvent by distillation under diminished pressure was taken up in ether, the solution was dried with sodium sulfate, and the ether was removed by distillation under diminished pressure. The product was dried at 100°C. in a high vacuum ($p = 0.05$ mm.) and analyzed as follows:

0.1082 gm. substance: 0.1824 gm. CO_2 and 0.0682 gm. H_2O .

0.1760 " " : 0.4134 " AgI (Zeisel).

$\text{C}_8\text{H}_{14}\text{O}_6$ (mol. wt. 206). Calculated. C 46.50, H 6.80, CH_3 30.10.

Found. " 45.97, " 7.05, " 31.0.

The product titrated as a lactone.

540 Rotation of Methylated Gluconic Acids

0.0863 gm. of substance was neutralized by 4.25 cc. of 0.1 N alkali, equivalent to a molecular weight of 203. It had the following optical rotation in aqueous alcohol.

$$[\alpha]_D^{20} = \frac{+ 1.64^\circ \times 100}{1 \times 2.81} = + 58.5^\circ$$

Sodium Salt.—0.4100 gm. substance was dissolved in 2.5 cc. N NaOH. This was heated at 80–90°C. for several hours and made up to 5 cc. with water.

$$[\alpha]_D^{20} = \frac{+ 3.62^\circ \times 100}{1 \times 8.2} = + 44.1^\circ$$

Free Acid.—4 cc. of the solution used for the previous rotation were pipetted into a 10 cc. flask, cooled with ice and alcohol, and neutralized with 2.25 cc. N HCl.

After 24 hrs.

$$[\alpha]_D^0 = \frac{+ 1.47^\circ \times 100}{2 \times 3.28} = + 22.5^\circ \qquad [\alpha]_D^{20} = \frac{+ 2.65^\circ \times 100}{2 \times 3.28} = + 40.4^\circ$$

The above procedure for determining the optical rotation of the salt and free acid was followed throughout.

3,5,6-Trimethyl Gluconic Lactone.

3,5,6-Trimethyl Monoacetone Glucose.—Monoacetone glucose in portions of 20 gm. was methylated with 125 gm. of methyl sulfate and 250 cc. of 30 per cent sodium hydroxide. The methylated sugar separated as an oil on the surface of the liquid. It was extracted with ether, dried with sodium sulfate, and the ether removed under reduced pressure. The syrup, 21 gm., was distilled. It boiled at 110°C., $p = 0.3$ mm., $n_D^{23} = 1.44914$.

It analyzed for the methoxyl as follows:

0.1098 gm. substance: 0.2842 gm. AgI (Zeisel).

$C_{12}H_{22}O_6$. Calculated. OCH_3 35.4.

Found. " 34.1.

3,5,6-Trimethyl Glucose.—The acetone sugar was hydrolyzed with 0.5 per cent hydrochloric acid and the trimethyl glucose which was isolated by the usual procedure was analyzed without previous distillation.

0.1102 gm. substance: 0.1992 gm. CO_2 and 0.0848 gm. H_2O .

$\text{C}_9\text{H}_{18}\text{O}_6$. Calculated. C 48.65, H 8.11.

Found. " 49.22, " 8.60.

3,5,6-Trimethyl Gluconic Lactone.—The trimethyl glucose was oxidized by bromine as already described. The hydrobromic acid was neutralized with the calculated amount of 1.0 N NaOH after a quantitative determination of the HBr by Volhard's method. The lactone was extracted with ether after the solvents had been removed under reduced pressure, and the ether solution treated as usual. The lactone which was obtained as a syrup was distilled and boiled at 155° , $p = 1$ mm. It analyzed as follows:

0.1186 gm. substance: 0.2114 gm. CO_2 and 0.0796 gm. H_2O .

0.1462 " " : 0.4806 " AgI (Zeisel).

$\text{C}_9\text{H}_{16}\text{O}_6$ (mol. wt. 220). Calculated. C 49.05, H 7.3, OCH_3 42.24.

Found. " 48.60, " 7.51, " 43.39.

The product titrated as a lactone.

0.0802 gm. of substance was neutralized by 3.55 cc. of 0.1 N alkali, equivalent to a molecular weight of 225.

It had the following optical rotation in aqueous alcohol.

After 24 hrs.

$$[\alpha]_{\text{D}}^{20} = \frac{+1.25^\circ \times 100}{1 \times 2.836} = +44.1^\circ \quad [\alpha]_{\text{D}}^{20} = \frac{+1.13^\circ \times 100}{1 \times 2.836} = +39.8^\circ$$

Sodium salt.

$$[\alpha]_{\text{D}}^{20} = \frac{+1.10^\circ \times 100}{1 \times 4.57} = +24.0^\circ$$

Free acid.

After 24 hrs.

$$[\alpha]_{\text{D}}^{20} = \frac{-0.23^\circ \times 100}{2 \times 1.88} = -6.3^\circ \quad [\alpha]_{\text{D}}^{20} = \frac{+0.20^\circ \times 100}{2 \times 1.828} = +5.4^\circ$$

2,3,5-Trimethyl Gluconic Lactone.—2,3,5-trimethyl glucoside which was obtained by fractional distillation of methylated glucosides and which analyzed for methoxy 52.3 per cent, was converted by the usual procedure into the free sugar. The trimethyl glucose was oxidized with bromine by the method already described. The lactone distilled at 142°C ., $p = 0.14$ mm., and analyzed as follows:

542 Rotation of Methylated Gluconic Acids

0.1066 gm. substance: 0.1904 gm. CO₂ and 0.0716 gm. H₂O.

0.1172 " " : 0.3726 " AgI (Zeisel).

C₉H₁₆O₆ (mol. wt. 220). Calculated. C 49.05, H 7.27, OCH₃ 42.24.

Found. " 48.70, " 7.51, " 41.96.

The product titrated as a lactone. 0.0934 gm. substance was neutralized by 4.20 cc. 0.1 N alkali, equivalent to a molecular weight of 222.

It had the following optical rotation in aqueous alcohol.

$$[\alpha]_D = \frac{+ 2.23^\circ \times 100}{1 \times 2.452} = + 90.8^\circ \quad \text{After 24 hrs.} \quad [\alpha]_D^{20} = \frac{+ 1.35^\circ \times 100}{1 \times 2.452} = + 55.0^\circ$$

Sodium salt.

$$[\alpha]_D^{20} = \frac{+ 1.57^\circ \times 100}{1 \times 2.440} = + 64.4^\circ$$

Free acid.

$$[\alpha]_D^0 = \frac{+ 0.75^\circ \times 100}{2 \times 1.952} = + 19.3^\circ \quad \text{After 24 hrs.} \quad [\alpha]_D^{20} = \frac{+ 0.92^\circ \times 100}{2 \times 1.952} = + 23.5^\circ$$

Tetramethyl Gluconic Lactone.—Tetramethyl glucose was oxidized with Br by the methods already described. The lactone distilled at 128°C., *p* = 0.8 mm., and analyzed as follows:

0.1056 gm. substance: 0.1986 gm. CO₂ and 0.0730 gm. H₂O.

0.1069 " " : 0.4300 " AgI (Zeisel).

C₁₀H₁₈O₆. Calculated. C 51.20, H 7.7, OCH₃ 53.00.

Found. " 51.18, " 7.73, " 53.44.

The lactone had the following optical rotation in aqueous alcohol.

$$[\alpha]_D^{20} = \frac{+ 2.95^\circ \times 100}{1 \times 2.78} = + 106.1^\circ \quad \text{After 24 hrs.} \quad [\alpha]_D^{20} = \frac{+ 1.45^\circ \times 100}{1 \times 2.78} = + 52.2^\circ$$

Salt.

$$[\alpha]_D^{20} = \frac{+ 3.36^\circ \times 100}{1 \times 4.4} = + 76.4^\circ$$

Acid.

$$[\alpha]_D^0 = \frac{+ 1.5^\circ \times 100}{2 \times 1.76} = + 43.0^\circ \quad \text{After 24 hrs.} \quad [\alpha]_D^{20} = \frac{+ 1.74^\circ \times 100}{2 \times 1.76} = + 49.5^\circ$$

Pentamethyl Gluconic Acid.

Calcium gluconate in portions of 20 gm. was methylated with 125 cc. of dimethyl sulfate and 250 cc. of 30 per cent sodium hydroxide. The product was made neutral to Congo red with sulfuric acid and concentrated under diminished pressure to about 150 cc., filtering occasionally from the deposited sodium sulfate. It was then acidified with sulfuric acid and extracted with ether on a continuous extractor. After extracting in this manner for several days the ether solution was dried with anhydrous sodium sulfate and the ether removed under diminished pressure.

In this manner approximately 50 per cent by weight of the calcium gluconate employed was recovered as a syrup. The syrup contained only partially methylated gluconic acid having a methoxy value of 38 to 40 per cent. It was nearly completely soluble in methyl iodide and could therefore be further methylated by the method of Irvine without recourse to any extraneous solvent. The methyl iodide-silver methylation was twice repeated, using 140 gm. of methyl iodide and 100 gm. of silver iodide for each methylation. The methylated syrup, which was recovered with but slight loss, then contained approximately 2.0 per cent of OCH_3 .

Further methylation by this method failed to increase the methoxy content. Likewise, after treatment with diazomethane, the product still analyzed for 62.6 per cent of OCH_3 . This syrup distilled fairly constant at 112°C ., $p = 0.5$, mm., and could not be fractionated.

15 gm. of this product were therefore hydrolyzed with an excess (105 cc.) of 1.0 N sodium hydroxide at $80\text{--}90^\circ$ for 2 hours.

The above solution then had $[\alpha] = +5.80^\circ$ and further heating for 1 hour did not change this value. The solution was therefore concentrated to 50 cc. under diminished pressure without undue application of heat and the product methylated with 80 cc. of dimethyl sulfate and 180 cc. of 30 per cent sodium hydroxide. The methylated product was recovered from this solution exactly as described above.

The syrup was fractionated and distilled mainly at 155°C ., $p = 1$ mm.

544 Rotation of Methylated Gluconic Acids

It analyzed as follows:

0.1346 gm. substance: 0.2428 gm. CO₂ and 0.0980 gm. H₂O.

0.0998 " " " : (Zeisel) 0.4308 gm. AgI.

C₁₁H₂₂O₇ (mol. wt. 266). Calculated. C 49.60, H 8.32, OCH₃ 58.2.
 Found. " 49.11, " 8.14, " 56.81.

The product titrated as an acid, 0.0882 gm. of substance being neutralized by 3.30 cc. of 0.1 N alkali equivalent to a molecular weight of 267.

Pentamethyl gluconic acid had the following optical rotation in water.

$$[\alpha]_D^{20} = \frac{+1.00^\circ \times 100}{1 \times 4.44} = +22.5^\circ$$

Sodium salt.

$$[\alpha]_D = \frac{+2.52^\circ \times 100}{1 \times 4.7} = +53.7^\circ$$

After neutralization after sodium salt.

$$[\alpha]_D = \frac{+0.85^\circ \times 100}{2 \times 1.88} = +22.5^\circ$$

BROMOLECITHINS.

I. FRACTIONATION OF BROMINATED SOY BEAN LECITHINS.*

BY P. A. LEVENE AND IDA P. ROLF.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Lecithins prepared from animal organs or from plants contain two saturated and several unsaturated fatty acids. It has been assumed that this fact indicated the existence of several lecithins each containing only two fatty acids. The alternative view would be the one postulating the structure of lecithins as polylecithides, containing in one molecule all the fatty acids isolated from lecithins.

A priori, the simpler structure seems the more probable one, but up to the present, there has been no experimental evidence advanced which would give certain preference to one of these theories over the other.

Attempts to fractionate the lecithins had been made by us before, but with little success. Recently it was decided to try to fractionate, not the free lecithins, but their bromo derivatives.

Several reasons suggested this method. First, our experience in separating cephalin from lecithin has shown that the task was more easily accomplished after the phosphatides had been hydrogenated. Second, it is known that the solubilities of the higher aliphatic bromo acids decrease with the increase in the number of bromine atoms. Thus hexabromostearic acid is much less soluble than tetrabromostearic acid.

Preparation of bromolecithins has been mentioned in patent literature, but the substances have not been utilized for theoretical purposes. The present investigation has been limited to lecithins extracted from soy beans.

* We wish to acknowledge our indebtedness to Dr. Bollmann of the Hanseatische Mühlenwerke for supplying the material used in this investigation.

When a solution of such lecithins in petroleic ether is treated with a solution of bromine in the same solvent, a product insoluble in this solvent is formed.

Before attempting fractionation of the bromolecithins, the fatty acids of the whole material were separated and analyzed. From the bromine content of the material it seemed probable that it contained in the main two brominated acids besides the saturated. In accordance with this assumption, it has been possible to isolate from this material and to identify hexabromostearic and tetrabromostearic acids.

This find encouraged the attempt to fractionate the bromolecithins. Indeed we have succeeded in separating the hexabromolecithin in pure form, and a tetrabromo derivative in a state sufficiently pure to warrant the assumption of the existence of an individual tetrabromolecithin. The dibromolecithin should be obtainable from the mother liquors of the insoluble bromolecithins.

Thus, the work on bromolecithins lends experimental support to the assumption that lecithins as isolated from animal and plant tissues are mixtures of individual lecithins each containing two fatty acids.

The nature of the saturated acids associated with each unsaturated acid will be the subject of the next investigation. The work will also be extended to lecithins prepared from animal tissues.

EXPERIMENTAL.

The mixture of lecithins used as intermediary material was isolated as a cadmium chloride salt from the lipid fraction of the soy bean in the manner described in a previous publication.¹ The lipid mixture, freed from cadmium chloride with ammonia, analyzed as follows:

0.1069 gm. substance: 0.2501 gm. CO₂, 0.1007 gm. H₂O, and 0.0084 gm. ash.

0.1945 gm. substance required (Kjeldahl) 2.63 cc. 0.1 N acid.

0.2931 " " : (fusion) 0.0410 gm. Mg₂P₂O₇.

¹ Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1924-25, lxii, 759.

2 gm. substance were hydrolyzed with 10 per cent HCl.

5 cc. required (Kjeldahl) 0.29 cc. 0.1 N acid.

2 " " (Van Slyke) 0.09 cc. N₂ at 22°C., 748 mm.

C₄₃H₈₆O₉NP. Calculated. C 65.26, H 10.95, N 1.77, P 3.92.

Found. " 63.80, " 10.54, " 1.89, " 3.88.

$$\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{3}{100}$$

Bromination of this material was accomplished most satisfactorily by dissolving the lipid in 10 parts of gasoline (boiling at 40–50°C.) and adding the bromine (dissolved in 5 parts of gasoline) at such a rate that the temperature of the reaction mixture could be kept below –5°. As the reaction proceeded, the brominated lipids gradually precipitated, giving rise to a characteristic color change in the solution. When the bromine was no longer decolorized the supernatant liquor was decanted, and the precipitated material was very thoroughly washed with fresh gasoline to remove any excess bromine. The yield of this crude material was theoretical. Finally the precipitate, which resembled lecithin in texture but was of a more silvery appearance, was dissolved in ether. From the ethereal solution a material was separated on standing at 0°C. which at this temperature seemed to consist of a light yellow, finely divided amorphous powder. On warming to room temperature, however, it coalesced to a typical lecithin-like mass, rather harder than the ordinary unsaturated lipid but of the same light reddish brown color. This fraction analyzed as follows:

No. 187. 0.200 gm. substance required (Kjeldahl) 1.95 cc. 0.1 N acid.

0.300 " " : (fusion) 0.0300 gm. Mg₂P₂O₇.

0.1342 " " : (Carius) 0.1128 gm. AgBr.

Hexabromostearyl stearyl lecithin, C₄₄H₈₄O₉NPBr₆.

Calculated. N 1.09, P 2.42, Br 37.45.

Found. " 1.36, " 2.72, " 35.77.

The ether-soluble fraction of the brominated lipids was precipitated by the addition of several volumes of gasoline and formed a shiny soft buttery mass, which was soluble in acetone, alcohol, and glacial acetic acid. It analyzed as follows:

No. 189. 0.2000 gm. substance required (Kjeldahl) 2.10 cc. 0.1 N acid.

0.3000 " " : (fusion) 0.0310 gm. Mg₂P₂O₇.

0.1296 " " : (Carius) 0.0988 " AgBr.

Tetrabromostearyl stearyl lecithin, $C_{44}H_{86}O_9NPBr_4$.

Calculated. N 1.24, P 2.76, Br 28.50.

Found. " 1.47, " 2.88, " 32.44.

To effect further fractionation, this material was again dissolved in ether, and again a small amount of precipitate could be separated which was insoluble in ether at 0° . The more soluble portion was fractionated by the gradual addition of gasoline. The fraction which remained soluble in a 50 per cent mixture of ether and gasoline at 0° was precipitated by the addition of a large excess of gasoline. This fraction analyzed as follows:

No. 199. 0.2170 gm. substance required (Kjeldahl) 2.55 cc. of 0.1 N acid.

0.3150 gm. substance: (fusion) 0.0356 gm. $Mg_2P_2O_7$.

0.1054 " " : (Carius) 0.0778 " AgBr.

Found. N 1.64, P 3.15, Br 31.41.

Bromo Acids from the Mixed Bromolecithins.

Hexabromostearic Acid.—40 gm. of the crude mixture of brominated lecithin were hydrolyzed for 8 hours with 10 parts of 10 per cent hydrochloric acid. The mixture of bromo and saturated fatty acids was separated by filtration, dissolved in ether, thoroughly washed with water, and dried. The total mixed acids weighed 28.5 gm. and had a bromine content of 29.03 per cent. (A mixture composed of 50 per cent stearic acid, 25 per cent hexabromostearic acid, and 25 per cent tetrabromostearic acid should have a bromine content of 29.16 per cent.) These crude acids were thoroughly extracted with gasoline and the insoluble bromo acid fraction was dissolved in methyl alcohol. From this solution, on cooling, a precipitate separated, which after recrystallization from methyl alcohol and thorough extraction with ether yielded 0.8 gm. of material melting at 180° , and giving the following analysis.

No. 191. 0.1045 gm. substance: 0.1120 gm. CO_2 and 0.0390 gm. H_2O .

0.1004 " " : (Carius) 0.1510 gm. AgBr.

Hexabromostearic acid, $C_{18}H_{30}O_2Br_6$.

Calculated. C 28.49, H 3.99, Br 63.26; m.p. = $180-181^\circ$.

Found. " 29.22, " 4.17, " 64.00.

When mixed with hexabromostearic acid obtained by brominating linolenic acid, no depression of the melting point was apparent.

Tetrabromostearic Acid.—The acids which remained dissolved in the methyl alcohol solution were precipitated with lead acetate, and the lead salts were suspended in toluene and decomposed. The addition of gasoline to the concentrated solution caused the immediate precipitation of an insoluble resinous material. On standing the liquor decanted from this gum deposited 2.5 gm. of the characteristic white crystals of tetrabromostearic acid. They gave the following analysis.

No. 192. 0.1032 gm. substance: 0.1354 gm. CO_2 and 0.0506 gm. H_2O .
0.0744 " " : (Carius) 0.0956 gm. AgBr.

Tetrabromostearic acid, $\text{C}_{18}\text{H}_{32}\text{O}_2\text{Br}_4$.

Calculated. C 36.01, H 5.38, Br 53.28; m.p. = 114° .

Found. " 35.77, " 5.48, " 54.69.

This material melted at $113\text{--}114^\circ$, and showed no depression of the melting point when mixed with tetrabromostearic acid prepared from linolic acid.

CONCLUSIONS.

1. Plant lecithin has been brominated.
2. From the bromolecithins a hexabromo and a tetrabromo derivative have been isolated.
3. This fractionation is regarded as evidence in favor of the theory viewing the ordinary lecithins as mixtures of several individual forms.

THE THIO-SUGAR FROM YEAST.

By P. A. LEVENE AND HARRY SOBOTKA.

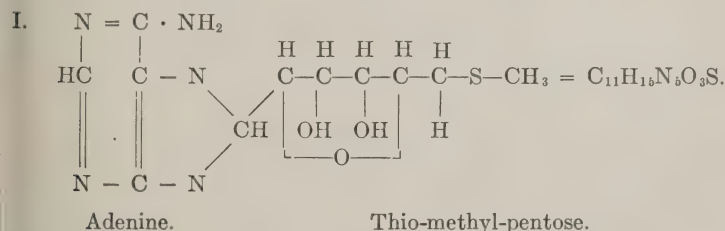
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 6, 1925.)

In 1912, Mandel and Dunham¹ discovered in the commercial yeast product "zymin" an adenine nucleoside of the elementary composition $C_{11}H_{15}N_5O_5$. They recognized the base as adenine but furnished little information as to the nature of the carbohydrate component.

Levene,² in 1924, in the course of work on brewers' yeast, incidentally isolated the substance discovered by Mandel and Dunham and corroborated their conclusions regarding the nature of the base. The sugar isolated from the nucleoside had the properties of a ketohexose, but differed from all known ketohexoses.

Suzuki, as far back as 1914, described a base of the elementary composition $C_9H_{12}N_4O_4$. At the time of its discovery, he failed to recognize the glucosidic nature of the substance. Very recently, however, Suzuki, in cooperation with Odake and Mori^{3,4} recognized the nucleosidic nature of the substance and made the most interesting discovery that the sugar contained sulfur in its molecule. In the German publication of their work, they consider as possible the first three of the following four structures. The fourth is contained in their Japanese publication.



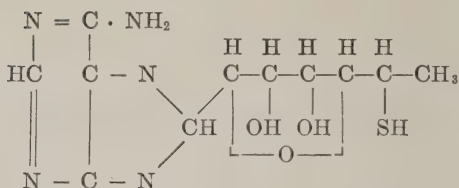
¹ Mandel, J. A., and Dunham, E. K., *J. Biol. Chem.*, 1912, xi, 85.

² Levene, P. A., *J. Biol. Chem.*, 1924, lix, 465.

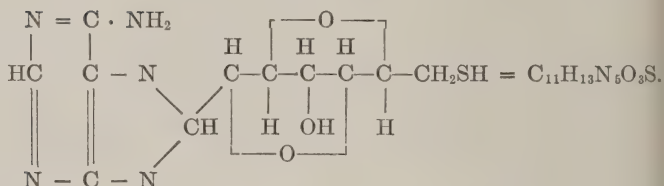
³ Suzuki, U., Odake, S., and Mori, T., *J. Agric. Chem. Soc. Japan*, 1924, No. 2.

⁴ Suzuki, U., Odake, S., and Mori, T., *Biochem. Z.*, 1924, cliv, 278.

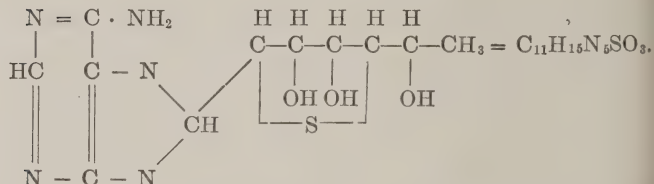
II.



III.



IV.



Suzuki and his coworkers, for some reason, did not associate their nucleoside with that of Mandel and Dunham. Had they done so, and had they taken into consideration the observations of Levene, they would have reduced the possibilities of the structure of their sulfo-sugar to a smaller number.

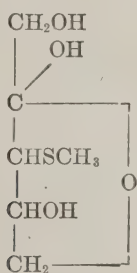
Since the molecular weight of sulfur is twice that of oxygen, it is evident that the carbon and hydrogen values for a hexose and for a sulfomethyl-pentose and the carbon and hydrogen and nitrogen values for their respective osazones are identical.

We have, therefore, tested the nucleoside prepared by one of us and found that it contained the same proportion of sulfur as the substance of Suzuki. We have also analyzed the old osazones in our possession and found that they likewise possessed the requisite amount of sulfur. Furthermore, the parabromophenylosazone described by Levene was prepared from the sugar after it had been exposed to the action of an excess of bromine during 96 hours. These data alone are sufficient to exclude first, the aldehyde structure accepted by Suzuki and his coworkers in all the

formulae and second, the presence of an $-SH$ group as in figures (II) and (III).

The experiments previously reported by one of us have now been repeated. Our present results are identical with those already recorded. We have further extended them; namely, first, we prepared an acetyl derivative by the Behrend (pyridine) method. The substance was purified by distillation and had the composition of the triacetyl derivative. Therefore, formula (IV) of Suzuki is definitely excluded. Second, by distillation of the sugar with hydriodic acid we demonstrated the presence of either an $-OCH_3$ or an $-SCH_3$ group.

Since the sugar forms an osazone very readily, it is likely by analogy with other 2-keto-sugars that the $-SCH_3$ (or $-OCH_3$) group is not attached to carbon atom 1. Hence, the choice is reduced to the following two structures.



V.



VI.

The position of the substituent group is for the present arbitrary, as it may be in position (4) as well.

The details of the configuration of the sugar will not be cleared up until the problem of Walden inversion receives an adequate solution.

EXPERIMENTAL PART.

The sugar used in these experiments consisted of a very thick syrup which was dried by removing the water by distilling at a pressure of 0.1 mm. The residue was taken up in alcohol and distilled as before. This operation was repeated several times, the ethyl alcohol being interchanged with methyl alcohol.

The final residue had the following rotation in methyl alcohol.

⁵ Kirpal, A., and Böhn, Th., *Ber. chem. Ges.*, 1914, xlvii, 1084.

THE SUGAR CONTENT OF BLOOD.

By BEN K. HARNED.

From the Department of Chemistry, College of Medicine, University of Tennessee, Memphis.)

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A new method, based upon the use of a more specific sugar reagent, has recently been proposed by Benedict¹ for the determination of blood sugar. This method gives values which are about 20 per cent lower than those obtained by the Folin-Wu procedure. Because of the fact that the blood contains several known reducing substances other than glucose, and probably other unidentified interfering compounds, it is generally accepted that the method which yields the lowest results, and at the same time satisfactorily recovers added glucose, should be regarded as the most accurate criterion of the blood sugar content.

The results obtained by Benedict have decided us to report a comparable investigation carried out during the last 2 years upon the determination of the blood sugar in a filtrate from which many, if not all, of the interfering blood components had been removed. Our findings are in definite agreement with those of Benedict.

In our procedure we precipitate the blood proteins by a slight excess of acid mercuric nitrate. Excess of mercury and unoxidized nitrogen are removed from the filtrate by successive precipitations with sodium bicarbonate and hydrogen sulfide. The final filtrate is substituted for the tungstic acid filtrate in the Folin-Wu method.

Detailed Procedure.

One volume of blood is diluted with eight volumes of water in an Erlenmeyer flask. While rotating the mixture one volume of mercuric nitrate solution² is added, a little at a time. The blood proteins are precipitated

¹ Benedict, S. R., *J. Biol. Chem.*, 1925, lxiv, 207.

² The mercuric nitrate reagent is prepared as follows: 348 gm. of mercuric nitrate and about 900 cc. of water are shaken in a liter flask for 10 to 15 minutes. Concentrated nitric acid is then added, a few cc. at a time, and finally drop by drop, until the mercuric nitrate is dissolved. The solution is then made up with water to a volume of 1 liter.

in a jelly-like mass. The flask is stoppered and shaken vigorously for several minutes to break up the precipitate, and the mixture is then filtered. The filtrate is water-clear and colorless. Solid sodium bicarbonate is added to the filtrate until effervescence ceases and the reaction is faintly alkaline to litmus. The resulting precipitate is removed by filtration through a double thickness of retentive paper. Dried and finely powdered potassium bisulfate is added to the filtrate until a drop of the mixture gives with tropaolin 00 on a spot plate the intermediate color shade. Traces of mercury are now removed from the solution by precipitation by hydrogen sulfide gas, which is first bubbled through water. After filtering off the mercuric sulfide, excess hydrogen sulfide is blown out of the filtrate by a current of air which passes first through a water wash bottle. The final solution is treated in exactly the same manner as the standard in the Folin-Wu procedure. The sugar standard employed is brought to the same reaction as the unknown by the addition of potassium bisulfate.

The following comments upon the procedure are appropriate.

1. Glucose added to the blood is quantitatively recovered.
2. The final filtrate is free of unoxidized nitrogen. As an illustration, we had one blood in our series with a non-protein nitrogen value of 281 mg. per 100 cc., as determined in the tungstic acid filtrate. When 10 cc. of the final filtrate obtained from the mercuric nitrate procedure upon this blood was subjected to a micro Kjeldahl determination, the distillate gave with Nessler's reagent no more color than a blank.
3. Sodium nitrate, added to a sugar standard in amount equal to the maximum concentration possible in a blood filtrate from the mercuric nitrate procedure, has no effect upon the color value of the standard in the Folin-Wu method.
4. The mercuric nitrate filtrate is made acid to tropaolin 00 to prevent fading of the subsequent blue color. This detail is the result of a chance observation, and we can offer no explanation of its effect other than the possibility that the higher acidity prevents the formation of traces of nitrites from nitrates during the hydrogen sulfide treatment. Color fading was the most troublesome and time-consuming problem of our work. Variations in the reaction of the sugar solution very appreciably affect the amount of reduction of the Folin-Wu alkaline copper reagent. With a little care, however, the reaction of the blood filtrate and the sugar standard may be adjusted to closer agreement by addition of potassium bisulfate than is usually true of filtrate and standard in the original Folin-Wu procedure. We have made many com-

comparative determinations of blood sugar by the original Folin-Wu method, and after making both the Folin-Wu filtrate and standard acid to tropaolin 00 by potassium bisulfate, with very satisfactory

TABLE I.

Comparative Values for Blood Sugar by the Folin-Wu Method, by the New Benedict Method, and by the Mercuric Nitrate Method.

Source.	Folin-Wu method.	Benedict method.	Mercuric nitrate method.	
			(1)*	(2)†
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Human.....	100		86	80
".....	113		99	92
".....	93		77	71
".....	87		71	66
".....	68		59	55
".....	121		107	100
".....	101		86	80
".....	98		84	79
".....	120		106	99
".....	115		101	93
Phlorhizinized dog.....	74		60	56
" ".....	73		48	45
Human.....	94	78	77	71
".....	94	82	79	74
".....	96	78	75	70
".....	81	70	72	68
".....	97	86	90	84
".....	81	70	72	68
".....	94	77	79	74
".....	82	75	71	66
" (diabetes).....	143	123	132	123
".....	66	57	48	45
".....	87	76	74	69
".....	103	91	97	91
".....	77	66	69	65
".....	120	97	102	95
".....	68	59	54	51
".....	108	89	88	82

* The values in this column are based upon comparison with a standard made acid by addition of a specified amount of potassium bisulfate.

† The values in this column are corrected for the average increment in color of the standard resulting from preliminary treatment with mercuric nitrate as in the case of the blood filtrate.

agreement. The solutions to which the specified amount of potassium bisulfate has been added are very close to 0.05 N acid by titration, a value which represents about one-fourteenth the neutralizing capacity of the Folin-Wu alkaline copper reagent.

5. Hydrogen sulfide was finally selected to remove from the filtrate the mercury which escapes sodium bicarbonate precipitation, after repeated trials of all other means that occurred to us. Zinc and metals above hydrogen in the electromotive series cannot be used for the purpose, probably because of the formation of nitrite from nitrate. A very small concentration of nitrite completely dissipates the final blue color in the Folin-Wu method. Copper and metals between hydrogen and mercury in the electromotive series appeared theoretically objectionable because of the possibility of disturbing the copper balance in the subsequent reaction, or oxidation of the sugar during removal of the mercury. Notwithstanding, several (possibly an inconclusive³ number) tests were made of copper in this connection, with such varying and uncertain results that this agency was abandoned in favor of hydrogen sulfide.

6. When the sugar standard is treated with the mercuric nitrate reagent, and the mercury removed as in the case of a blood filtrate, its color-producing value in the Folin-Wu method is slightly higher than for a standard only made acid by potassium bisulfate. We have made a large number of such comparisons, and have found that the mercury-treated standards read between 13.5 and 14.5 mm. when the non-mercury-treated standard is set at 15 mm. The average comparison is 14.0 to 15.0 mm. This correction has been taken into account in one calculation of our results, as shown in Table I. We are not certain, however, that this correction brings us nearer to the true blood sugar values. When the filtrate

³ A paper by Bierry and Moquet (Bierry, H., and Moquet, L., *Compt. rend. Soc. biol.*, 1924, xc, 1316) has just come to our attention, in which paper is reported an adaptation of the Folin-Wu procedure to blood filtrates from mercuric nitrate precipitation. Bierry and Moquet employ copper to remove the final traces of mercury from the filtrates. They do not refer to any of the difficulties encountered by us in obtaining filtrates which would not show fading of the color developed, nor do they report any comparative values by their method and the original Folin-Wu method. A table, which does not show the usual proportionality between colorimetric readings and glucose content, is supplied for translation of the results.

from the mercuric nitrate precipitation of blood is treated with sodium bicarbonate appreciably more mercury escapes precipitation than is the case with a sugar standard. This results in a more considerable precipitate of mercuric sulfide in the case of the blood filtrate, when hydrogen sulfide is subsequently introduced; the precipitate is better flocculated and is completely removed by filtration. In the case of the sugar standard treated with mercuric nitrate there is a pronounced tendency for the mercuric sulfide precipitate to pass into the colloidal state, so that the final filtrates in this case are almost invariably slightly tinged with brownish, colloiddally dispersed mercuric sulfide. We can find no other explanation for the slight enhancement in color-producing value of the standard.

7. We would emphasize that the above described procedure is not suggested or recommended as a routine method, because of the laborious and exacting technique involved. We have employed it with the one purpose of seeking the absolute concentration of glucose in the blood.

DISCUSSION.

In Table I are given the results of comparative sugar analyses of twenty-eight blood samples, by the original Folin-Wu method, by the new Benedict method, and by the writer's procedure in which the Folin-Wu reactions are applied to a blood filtrate free of unoxidized nitrogen and probably the most important interfering, non-glucose compounds. The very close agreement between the results by the last two methods supports Benedict's claim of greater specificity for his new blood sugar method.

For the entire series the results by the Folin-Wu procedure average 17 per cent higher than those by the mercuric nitrate method; or, if a somewhat uncertain correction factor is applied, the difference between the two methods is 25 per cent. Comparing the last sixteen bloods, the Folin-Wu results average 17 per cent higher than the Benedict values.

It is of interest to refer briefly, in conclusion, to several sugar analyses of tissue extracts, by the Folin-Wu and mercuric nitrate methods. Two lots each of muscle and liver tissues were extracted with boiling water, and the proteins precipitated by colloidal iron. After concentration of the protein-free filtrates these were treated

exactly as blood. The Folin-Wu procedure gave for the two muscle extracts 48 and 41 mg. of glucose per 100 cc., respectively; the corresponding mercuric nitrate values were, 19 and 19 mg. For the two liver extracts the Folin-Wu values were 55 and 106 mg. of glucose per 100 cc.; the corresponding values by the mercuric nitrate method were 56 and 107 mg.

SUMMARY.

The Folin-Wu reactions for blood sugar have been applied to blood filtrates from mercuric nitrate precipitation. Such filtrates contain nitrogen only in the form of nitrate, and are thus free of many, if not all, of the interfering, non-sugar blood components. The Folin-Wu values for blood sugar average from 17 to 25 per cent higher than those found by the modified procedure. The results are in accord with those given by the new Benedict method for blood sugar.

A METHOD FOR THE DETERMINATION OF TOTAL SULFATES IN TISSUES.

BY W. DENIS AND STELLA LECHE.

*From the Laboratory of Physiological Chemistry of the School of Medicine,
Tulane University, New Orleans.)*

(Received for publication, July 6, 1925.)

Our knowledge of the sulfate content of animal tissues is extremely limited (1), for while many analyses have been published for total sulfur in foods and in animal and plant tissues, these determinations were made by ashing and include the sulfur present in combination in the protein molecule. This scarcity of data is, in part at least, due to lack of suitable analytical technique. The only method which can be said to give figures for the non-protein sulfate of tissues is the procedure described by Koch and Upson (2), which is extremely laborious, and has seen little use in the 16 years which have passed since its publication.

As a preliminary to an investigation of the distribution in the body of injected sulfates we have carried on a large amount of experimental work in an effort to devise some procedure of simplicity and accuracy sufficient for the problems in hand.

The method finally adopted consists in the digestion of the finely minced tissue by heating with dilute hydrochloric acid in an autoclave, a procedure which causes the proteins to go more or less completely into solution. The precipitation of the sulfate is effected by means of barium chloride, followed by filtration and weighing of the barium sulfate in the usual manner.

The details of the procedure are as follows:

Place 10 gm. of tissue which has been finely divided by passing through meat grinder in a large (25 × 200 mm.) Pyrex test-tube, and add 50 cc. of approximately normal hydrochloric acid. Then close the top of the test-tube by means of a small watch-glass held in place by a square of tin-foil and heat in the autoclave at 200°C. for 1 hour. We have throughout this

work used a so called "chemical" autoclave of about 5 liters capacity, capable of withstanding a pressure of 60 atmospheres. If such an instrument is not available digestion may be brought about by heating at 150°C. for 2 hours in a bacteriological autoclave. At the end of this period of heating the protein has completely passed into solution, but as a rule a layer of partially decomposed fat will be found on the surface of the liquid.

TABLE I.

Results Obtained on the Recovery of Potassium Sulfate Added to Beef and Dog Tissues.

Experi- ment No.	Tissue.	Per 100 gm. tissue.				Recovery
		Total sulfates present in tissues.	Sulfates added.	Total sulfates found.	Total sulfates recovered.	
		mg.	mg.	mg.	mg.	per cent
1	Lung (dog).	1.0	230.0	238.0	237.0	103.0
2	" (")	1.0	230.0	233.0	232.0	100.8
3	Liver (")	21.2	230.0	253.2	232.0	100.8
4	" (")	20.2	230.0	251.2	231.0	100.4
5	" (beef).	2.3	69.0	73.8	71.5	103.6
6	" (")	2.3	69.0	73.8	71.5	103.6
7	Muscle (dog).	0.87	230.0	221.7	220.5	95.9
8	" (")	0.87	230.0	224.87	224.0	97.4
9	" (beef).	Traces.	92.0	88.6	88.6	96.4
10	" (")	"	92.0	87.5	87.5	95.2
11	" (")	"	138.0	141.5	141.5	102.5
12	" (")	"	138.0	134.5	134.5	97.5
13	Kidney (dog).	9.5	230.0	239.0	229.5	99.8
14	" (")	4.39	230.0	241.39	237.0	103.6
15	" (")	4.39	230.0	234.89	230.5	100.2
16	" (beef).	3.8	46.0	53.3	49.5	107.6
17	Heart (dog).	1.58	230.0	235.08	233.5	101.0
18	" (")	1.58	230.0	237.58	236.0	102.6
19	Brain (beef).	11.1	138.0	148.9	137.8	99.5
20	" (")	11.1	138.0	151.2	140.1	101.5

The contents of the tube are transferred quantitatively to a 100 cc. volumetric flask by means of distilled water, the flask filled to the mark, and after thoroughly mixing, the liquid is transferred to two 50 cc. centrifuge tubes and centrifuged for about 5 minutes to precipitate any coarse particles of undissolved material which would later clog the filter. The centrifuge liquid is then filtered by suction through a Gooch crucible provided with thin asbestos mat, 40 cc. of the filtrate are transferred to a 250 cc. beaker, and the sulfates precipitated by the addition of 10 cc. of 5 per cent barium chloride solution, added drop by drop from a burette. The liquid is the

heated to boiling, allowed to stand overnight, and after transfer of the precipitate to a tared Gooch crucible provided with an asbestos mat. The barium sulfate is washed, dried, and ignited to constant weight in the usual manner.

In Table I we have collected the results of a number of analyses carried out by the above method on the tissues of normal dogs, and also on portions of the same tissues to which had been added known amounts of potassium sulfate.

The question may well be asked as to whether results obtained by autoclave digestion represent only preformed sulfates, or whether they include fractions split off from the various little known sulfur compounds, which exist in tissues and which may subsequently be transformed into sulfate by oxidation. Of the sulfur compounds known to be present in tissues cystine may be considered to be the only one found in any appreciable amount. To determine the behavior of cystine under the experimental conditions employed the following experiment was carried out.

0.4326 gm. of pure cystine prepared from wool was heated with 50 cc. of normal hydrochloric acid for 1 hour in the autoclave at a temperature of 100°C. At the end of this time the volume of the solution was made up to 100 cc. and two 50 cc. portions of this liquid were treated with barium chloride solution, heated again to boiling, and allowed to stand for 24 hours. At the end of this time no trace of a precipitate of barium sulfate was visible, even after centrifuging the solutions.

As a result of this work we feel justified in concluding that no cystine sulfur is transformed into sulfate in the course of our determinations. Cysteine and glutathione would presumably be more unstable than cystine; the existence of the former compound as a primary decomposition product by protein has however never been definitely proven, while the latter substance (3) is believed to be present in such small amounts that it would appear to be unimportant in connection with this problem. Until direct evidence can be obtained regarding the possible interference of unstable sulfur compounds present in tissues we feel justified in regarding the values found as representing the content of total sulfates in tissues.

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ON THE DISTRIBUTION OF INJECTED SULFATES IN TISSUES.

BY W. DENIS AND STELLA LECHE.

*(From the Laboratory of Physiological Chemistry of the School of Medicine,
Tulane University, New Orleans.)*

(Received for publication, July 6, 1925.)

In a series of observations made some years ago (1), it was found that the sulfate content of nephritic blood was frequently much increased, and that this rise was in some cases unaccompanied by a retention of any of the other inorganic constituents. Further experiments (2), made on dogs and on rabbits in which magnesium sulfate, sodium sulfate, magnesium chloride, sodium chloride, and sodium phosphate were administered by the intestine and by intravenous injection, and the subsequent excretion of these salts followed by means of blood analysis, were believed to indicate a selective retention on the part of the kidney for the sulfate ion which in one case was found to accumulate in the serum to a value of several thousand per cent of its initial concentration.

These results on sulfate retention have been confirmed by Barkus (3), who believed, however, that injected sulfates are retained, and probably stored in tissues other than blood, as his results indicate that they reappear only after a long period, and then disappear suddenly from the blood stream.

Undoubtedly a study of the blood alone cannot furnish evidence regarding the retention of any substance by tissues. To prove this point it is necessary to furnish data regarding the concentration of the substance under investigation, in tissues, blood, urine, and possibly also in intestinal contents. With this point in view we have therefore reinvestigated the question of the fate of sodium sulfate administered by intravenous injection of hyperosmotic solutions.

The experimental methods used were as follows:

Dogs were used as experimental animals. In every case 60 mg. of morphine sulfate were injected subcutaneously about 1 hour before the administration of ether was started. After anesthesia had been induced a tracheal cannula was inserted and connected to an apparatus for the mechanical administration of ether, the internal carotid artery, and the external jugular vein were then

TABLE I.
Normal Sulfate Content of Tissues.

Tissue.	S per 100 gm. of tissue.	Animal.
	Sulfate content.	
Lung.	1.0 mg. S per 100 cc.	Dog.
	1.9 " " " 100 "	"
	10.3 " " " 100 "	"
Liver.	20.2 " " " 100 "	"
	21.2 " " " 100 "	"
Muscle.	0.87 " " " 100 "	"
	12.1 " " " 100 "	"
	9.1 " " " 100 "	"
Kidney.	4.39 " " " 100 "	"
	9.5 " " " 100 "	"
Heart.	1.58 " " " 100 "	"
	5.1 " " " 100 "	"
Brain.	12.0 " " " 100 "	"
	14.0 " " " 100 "	"
	11.2 " " " 100 "	"
Muscle.	Traces only.	Beef.
Liver.	2.3 mg. S per 100 cc.	"
Kidney.	3.8 " " " 100 "	"
Brain.	11.1 " " " 100 "	"

bared, the former to serve for the obtaining of samples of blood and the latter for the introduction of the salt solutions. Urine collections were made by means of a catheter which was kept in the bladder during the entire experiment. Throughout the experiment the animal was kept warm by means of an electrically heated animal holder. The chemical determinations were made by the following methods. Sulfates in tissues by the method described in the preceding paper; sulfates in blood by the method described below; sulfates in urine by the procedure of Folin (4)

chlorides in urine by the Volhard method; and sulfates in blood were determined gravimetrically as follows:

To 25 cc. of citrated blood are added 55 cc. of distilled water and 20 cc. of 20 per cent trichloroacetic acid. The mixture is well shaken, let stand for 1 hour, and then filtered. To 25 cc. of the clear filtrate are added 75 cc. of water and 20 cc. of 5 per cent barium chloride solution. After standing overnight the precipitate is transferred to a tared Gooch crucible provided with an asbestos mat, washed, dried, ignited to constant weight, and weighed.

As a preliminary to our injection experiments we have made determinations of inorganic sulfates in a considerable number of samples of dog and of beef tissue. Some of the results obtained in this work are collected in Table I. As will be seen, there is considerable variation in the values obtained on tissues from different animals, in fact it is impossible to draw any conclusions regarding the localization of sulfates in any tissues from these figures. The values for the kidney and brain are it is true uniformly high, but the results obtained from the former tissue can be explained on the basis of unavoidable inclusion of traces of urine, while in the case of the brain the possibility of the high figures being due to the decomposition of some unstable sulfur-containing lipid cannot be ignored.

The available data regarding the content of inorganic sulfates in tissue are extremely scanty. Lee, Scott, and Colvin (5) have reported that the average inorganic sulfur content of four muscles of the cat amounted to 69 mg. per 100 gm. of fresh tissue. While Koch and Koch (6) found 7.7 mg. of inorganic sulfur per 100 gm. of brain tissue of the white rat, we have been unable to find any references to work done on any mammalian tissues other than the above.

Experiment 1.—Mar. 10, 1925. Dog 1, male, weight 7.2 kilos. Under morphine and ether anesthesia, injected into the external jugular vein 200 cc. of a warm 10 per cent solution of Na_2SO_4 (equivalent to 0.6075 gm. of S per kilo of body weight). Time of injection 9.30 to 9.36 a.m. 50 cc. samples of blood were taken from the carotid artery at 9.28 and at 9.38 a.m.; at 9.50 respiration ceased. The tissues were then immediately removed, finely minced, and the sulfate content thereof was determined.

Results.—

Blood before injection.....	1.0 mg. S per 100 gm.
“ 2 min. after injection.....	283.8 “ “ “ 100 “

Spleen.....	15.1 mg. S per 100 gm.
Muscle.....	23.0 " " " 100 "
Brain.....	28.0 " " " 100 "
Heart.....	100.2 " " " 100 "
Liver.....	133.8 " " " 100 "
Kidney.....	243.5 " " " 100 "
Lung.....	226.3 " " " 100 "

Experiment 2.—Mar. 31, 1925. Dog 2, female, weight 15.2 kilos. Under morphine and ether anesthesia, injected into the external jugular vein 200 cc. of a 10 per cent solution of Na_2SO_4 (equivalent to 0.28 gm. of S per kilo of body weight). Time of injection 9.28 to 9.40 a.m. 50 cc. samples of blood were taken from the internal carotid artery at 9.25 (before injection), at 9.41, and at 11.40 a.m. After the last sample of blood was drawn, the carotid cannula was opened and the dog allowed to become practically desanguinated before the removal of the tissues.

Urine was collected during the experiment by means of a catheter tied in the bladder. The volume collected amounted to 550 cc. and when analyzed for total sulfate was found to contain 1.560 gm. of S.

Results.—

Blood before injection.....	1.7 mg. S per 100 gm.
" 1 min. after injection.....	100.7 " " " 100 "
" 2 hrs. " "	23.29 " " " 100 "
Spleen.....	3.7 " " " 100 "
Muscle.....	3.0 " " " 100 "
Brain.....	16.5 " " " 100 "
Heart.....	3.1 " " " 100 "
Liver.....	11.9 " " " 100 "
Kidney.....	39.7 " " " 100 "
Lung.....	12.3 " " " 100 "

Experiment 3.—Apr. 7, 1925. Dog 3, male, weight 19.5 kilos. Under morphine and ether anesthesia, injected into the external jugular vein 200 cc. of a 10 per cent solution of sodium sulfate (equivalent to 0.225 gm. of S per kilo of body weight). Time of injection 9.44 to 9.52 a.m. 50 cc. samples of blood were taken from the carotid artery at 9.37 (before the injection), at 9.54, and at 11.48 a.m. After the last sample of blood was taken the carotid cannula was opened and the dog allowed to become desanguinated before removal of the tissues.

900 cc. of urine, collected during the experiment, were found to contain 2.566 gm. of total sulfate expressed as sulfur.

Results.—

Blood before injection.....	2.6 mg. S per 100 gm.
" 2 min. after injection.....	94.08 " " " 100 "
" 1 hr. and 56 min. after injection....	17.4 " " " 100 "
Spleen.....	7.6 " " " 100 "
Muscle.....	1.34 " " " 100 "
Brain.....	10.9 " " " 100 "

Heart.....	0.7 mg. S per 100 gm.
Liver.....	8.1 " " " 100 "
Kidney.....	28.0 " " " 100 "
Lung.....	8.8 " " " 100 "

Experiment 4.—Apr. 7, 1925. Dog 4, male, weight 19.5 kilos. Injected into the external jugular vein, 300 cc. of a 10 per cent sodium sulfate solution (equivalent to 0.33 gm. of S per kilo of body weight). Time of injection 9.17 to 9.37 a.m. 50 cc. samples of blood were taken from the carotid artery at 9.10, 9.38, and 11.37 a.m. After the last sample of blood was taken, the dog was allowed to bleed from the carotid artery until dead.

1200 cc. of urine collected from the bladder during the period of the experiment was found to contain 4.09 gm. of total sulfates calculated as S.

Results.—

Blood before injection.....	1.44 mg. S per 100 gm.
" 1 min. after injection.....	112.2 " " " 100 "
" 2 hrs. " " ".....	25.7 " " " 100 "
Spleen.....	3.04 " " " 100 "
Muscle.....	4.2 " " " 100 "
Brain.....	17.3 " " " 100 "
Heart.....	4.3 " " " 100 "
Liver.....	15.5 " " " 100 "
Kidney.....	87.0 " " " 100 "
Lung.....	26.4 " " " 100 "

The results obtained in Experiments 1 to 4 would appear to us to indicate that there is relatively little absorption of sulfate by the tissues, a conclusion which has been suggested by the work of other investigators (7). In Experiment 1, in which the dosage amounted to 0.60 gm. of S per kilo, and where only 100 cc. of blood were removed, it will be noted that the sulfate content of all of the tissues is much increased, this increase being most striking in those structures which are extremely vascular, such as the liver. We are inclined to believe that this increase is due to a large extent to the blood content of the organs and not to any retention of the salt by the tissues themselves, although it must be admitted that conclusive experimental proof of this interpretation is lacking.

In Experiments 2 to 4 we were compelled to lower the dosage considerably in order to keep our animals alive for any length of time, and in these experiments we have allowed the animal to be drained of blood by opening the carotid artery. In the case of these dogs it will be noted that the sulfate content of tissues was seldom if ever greater than the sulfate content of the sample of

blood taken just before death, while frequently it was much smaller.

SUMMARY.

In a series of experiments in which hypertonic solutions of sodium sulfate were administered to dogs by intravenous injections it was found by analysis of the blood, muscles, and viscera that there is little absorption of the sulfate ion by the tissues, although even 2 hours after injection the sulfate content of the blood may still be ten times its initial value.

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IS THE ANTIRACHITIC FACTOR OF COD LIVER OIL, WHEN MIXED WITH GROUND GRAINS, DE- STROYED THROUGH STORAGE?*

By E. B. HART, H. STEENBOCK, AND S. LEPKOVSKY.

(From the Department of Agricultural Chemistry, University of Wisconsin,
Madison.)

PLATE 1.

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Possession of the fact that cod liver oil has potent antirachitic properties has led to its direct mixing with foods designed for human consumption, particularly infant foods, and also with feeds intended for animal nutrition, especially poultry feeds. Such practice has, of course, raised the question of the permanency of the antirachitic properties of this mixture after several months of storage. Results published by Dunn (1) indicated that the antirachitic properties of cod liver oil had disappeared when mixed with granulated starch and stored for 6 months at 50°F. in corked bottles. Dunn observed what he called rickets in young chicks receiving the above mixture as a supplement to a basal ration, as early as the 20th day. It is very significant that in his experiments Dunn used a ration composed of skimmed milk, 97 parts of *white* corn, 2 parts of calcium carbonate, and 1 part of common salt and to which the starch-cod liver oil mixture was added. This basal ration was not only deficient in antirachitic properties, but was deficient in vitamin A. The fact that the cod liver oil-starch mixture had lost its vitamin A content, as shown by rat experiments conducted by Osborne (2), suggested the possibility that the results secured by Dunn were complicated by a vitamin A deficiency. Dunn's statement that the chicks suffered from rickets was supported only by growth records and by statements as to the birds' general condi-

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tion such as unsteady gait, lameness, swelling of joints, etc. Unless the diet is known to be sufficiently rich in vitamin A it is not safe to take the symptoms of unsteady gait and lameness as absolute criteria of rickets even if they are cured by the administration of cod liver oil. As is well known cod liver oil contains both vitamin A and vitamin D and resumption of growth and well being may have been due in these cases to either vitamin, when cod liver oil was administered.

Further, it is now known that vitamin A in cod liver oil is less stable to oxidative conditions than is the antirachitic factor. In fact accumulating evidence indicates that vitamin D is a fairly stable substance as compared to vitamin A. That it may be destroyed under certain conditions is, of course, admitted, but generalizations in respect to the conditions for its destruction cannot as yet be made. For example, it is known that cod liver oil stored in colorless glass bottles at room temperature and exposed to the light through a window will lose its vitamin A content in the course of 3 months; while its antirachitic properties are apparently undiminished. Storage in a brown bottle under the same conditions will result in much less destruction of vitamin A and in no loss of vitamin D (3). Further, it has been observed that vitamin D either in cod liver oil or as activated olive oil (4) when allowed to stand at room temperature in bottles kept in the dark was potent in its effects at the end of 10 months. In view of all the facts it seemed desirable to reinvestigate the stability of vitamin D of cod liver oil when stored with a grain mixture.

EXPERIMENTAL.

It was planned to do this work with baby chicks and to use a ration well supplied with vitamin A but poor in vitamin D. For this purpose a basal ration consisting of yellow corn 97, calcium carbonate 2, and sodium chloride 1 was ground up on November 15, 1924, in portions of 150 pounds each. One portion was left unsupplemented; to the second portion cod liver oil, constituting 0.5 per cent of the mass, was added and well mixed in; to the third portion 1.0 per cent of cod liver oil was added and also well mixed in.

The above food mixtures provided for three separate experi-

ments: a check, 0.5 per cent stored cod liver oil, and 1 per cent stored cod liver oil. To check the work further a sufficient supply of the basal ration was prepared and reserved for use at the actual time of the feeding tests, when a portion was mixed every 10 days with 0.5 per cent and 1 per cent of the same cod liver oil used in the storage experiments, but which had been kept in a tin container in mass and in a dark room; presumably under such conditions of storage, vitamin D had not appreciably decreased in the time involved.

In the three storage experiments the basal ration, or the basal ration supplemented with cod liver oil, was stored in round galvanized iron cans approximately 30 inches high and 20 inches in diameter. The cans and contents were kept at room temperature, about 70°F., and covered with a loosely fitting galvanized iron cover. No great circulation of air in the mass was possible, although over the upper surface free circulation could occur.

When these rations were fed, free access to skimmed milk was allowed. This skimmed milk was obtained from cows fed ordinary winter rations but kept indoors out of direct sunlight. No water was given. The chicks used were Barred Rocks taken when 1 day old. Shavings were allowed as litter in our experimental pens. Ten chicks were placed in each group. The plan was to keep the chicks 5 to 6 weeks on these rations when they would be killed for microscopic and chemical examination of the bones to determine the calcifying power of the ration. It was known to us from preliminary experiments that our basal ration of 97 parts of yellow corn, 2 parts of calcium carbonate, 1 part of common salt, and skimmed milk *ad libitum* would produce a distinctly rachitic condition in the baby chick in 5 weeks when kept out of direct sunlight or ultra-violet light. This rachitic condition could be recognized by the shambling gait, ruffled feathers, and frequent squatting of the birds provided vitamin A was plentifully supplied, but more closely by a low ash content of the alcohol-extracted bones, 35 to 40 per cent, and microscopically by the wide uncalcified area of proliferating cartilage of the tibia. In addition the chicks were weighed weekly. At 5 weeks of age the normal tibia of the chick contains 45 to 50 per cent of ash.

The rations were tested for their calcifying power after 3

months of storage and after 6 months of storage. At the end of 5 or 6 weeks on the ration the chicks were killed; the tibiae were removed and dried at 50°C. for 24 hours; the bones were crushed and subjected to hot 95 per cent alcoholic extraction for 36 hours. They were then ashed in an electric furnace. For microscopical examination the freshly removed tibia was immediately placed in 10 per cent formalin and kept there until used. For the silver nitrate test the bones were removed from the formalin; washed with water; the distal ends split and the section placed in 1.5 per cent silver nitrate solution for 1 minute; then removed to water and exposed to light for direct observation. For microscopical examination the tibia was first placed in 10 per cent formalin for 3 to 5 days, next washed with water and the proximal end cut off and split; these ends were then placed for 12 hours in 75 per cent alcohol and next decalcified in approximately 10 per cent nitric acid. The decalcifying solution was changed daily for 7 to 10 days. The preparations were again passed through 75 per cent alcohol and finally through absolute alcohol. After this treatment they were placed in a 50-50 solution of absolute alcohol and xylene for 2 hours, then transferred to pure xylene and left until translucent. They were then embedded, sectioned, and stained with hemotoxylin.

In order to save space data in respect to the ash content of the bones and silver nitrate tests are given for only a limited number of chicks from each group. In Table I the record is for the 3 months storage and the freshly admixed cod liver oil. In Table II the record is for 6 months of storage and for the rations containing the fresh cod liver oil.

It will be seen from Table I and Table II that the ash content of the tibia in the check group is low—the average being less than 40 per cent. The silver nitrate test also revealed this situation, invariably showing a wide uncalcified area of proliferating cartilage. In all of the other groups receiving either the stored material containing the cod liver oil as 0.5 per cent or as 1 per cent of the mass or as fresh cod liver oil in the same proportions the calcifying power of the ration was greatly improved. In fact, the calcifying power of the stored ration appeared to be just as good as that of the freshly made up ration. Further, the higher level of cod liver oil (1 per cent) appeared to be slightly more

TABLE I.

Cod Liver Oil Storage for 3 Months. Record of the Ash Content of Tibia and Silver Nitrate Tests.

Control.				
No.	Ash.	Weight.	Age.	Calcification AgNO ₃ test.
	<i>per cent</i>	<i>gm.</i>	<i>days</i>	
132	38.1	140	42	Poor.
133	36.7	150	42	Very poor.
137	40.2	175	42	Poor.
138	36.6	175	42	"
139	42.5	115	42	"
Average.....	38.8			
0.5 per cent cod liver oil. Stored 3 months before feeding.				
141	48.8	205	42	Good.
143	41.4	210	42	Poor.
145	47.6	210	42	Good.
147	49.5	160	42	"
150	44.7	220	42	Moderate.
Average.....	46.4			
1 per cent cod liver oil. Stored 3 months before feeding.				
151	50.6	240	42	Good.
154	50.7	395	42	"
155	50.9	210	42	"
157	51.5	190	42	"
158	47.2	215	42	"
Average.....	50.1			
0.5 per cent fresh cod liver oil. Mixed into ration every 8 days.				
161	48.6	190	42	Good.
165	44.6	230	42	"
167	42.4	230	42	Moderate.
168	44.3	210	42	"
169	44.1	175	42	"
Average.....	44.8			
1 per cent fresh cod liver oil. Mixed into ration every 8 days.				
172	39.4	205	42	Very poor.
175	49.8	235	42	Good.
178	50.6	260	42	"
179	49.5	245	42	"
180	48.9	240	42	"
Average.....	47.6			

TABLE II.

Cod Liver Oil Storage for 6 Months. Record of the Ash Content of Tibia and Silver Nitrate Tests.

Control.				
No.	Ash.	Weight.	Age.	Calcification AgNO ₃ test.
	<i>per cent</i>	<i>gm.</i>	<i>days</i>	
5901	41.1	105	35	Poor.
5902	38.7	180	35	Very poor.
5904	40.4	175	35	" "
5908	39.1	110	35	" "
5909	42.2	115	35	" "
5910	39.6	115	35	" "
Average.....	40.1			
0.5 per cent cod liver oil. Stored 6 months before feeding.				
5912	47.6	190	35	Good.
5913	41.5	175	35	Poor.
5914	47.6	175	35	Good.
5919	48.1	140	35	"
5920	47.4	140	35	"
Average.....	46.4			
1 per cent cod liver oil. Stored 6 months before feeding.				
5921	46.6	135	35	Good.
5922	47.2	175	35	"
5923	48.8	125	35	"
5924	46.4	130	35	"
5925	43.8	125	35	Moderate.
5927	47.8	240	35	Good.
5929	47.9	135	35	"
5930	47.4	125	35	"
Average.....	46.9			
0.5 per cent fresh cod liver oil. Mixed into ration every 8 days.				
5931	48.1	130	35	Good.
5932	42.2	135	35	Moderate.
5934	47.5	135	35	Good.
5935	43.5	130	35	Moderate.
5936	45.1	130	35	"
5937	48.6	110	35	Good.
5938	47.6	130	35	"
5939	40.0	180	35	Moderate.
Average.....	45.7			

TABLE II—*Concluded.*

1 per cent fresh cod liver oil. Mixed into ration every 8 days.

No.	Ash.	Weight.	Age.	Calcification AgNO ₃ test.
	<i>per cent</i>	<i>gm.</i>	<i>days</i>	
5941	48.3	170	35	Good.
5942	50.1	120	35	"
5944	51.2	190	35	"
5945	54.9	120	35	"
5946	45.6	150	35	"
5947	48.6	170	35	"
Average	49.7			

effective than the 0.5 per cent. However, there were individual exceptions to this rule. The ash content of the tibia of all of the groups receiving either stored cod liver oil or freshly admixed cod liver oil ran from 7 to 10 per cent. higher than the check group. The birds of the check group showed a distinct rachitic condition at the end of 5 weeks as exhibited by the shambling walk and squatting posture and gave a good exhibition of "leg weakness." None of the other groups exhibited this phenomenon.

Photomicrographs¹ of the proximal ends of the tibia from a representative from the check group, 0.5 per cent stored cod liver oil group, and the 0.5 per cent freshly admixed cod liver oil group, at 6 months of storage, are shown in Figs. 1, 2, and 3.

SUMMARY.

Cod liver oil mixed with ground grains and stored in cans at room temperature retained its calcifying power for at least 6 months.

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¹ The authors wish to express their appreciation to Mr. Henry Stevens for technical assistance in the preparation of the microscopical sections.

EXPLANATION OF PLATE 1.

Sections of the proximal ends of the tibia of chicks receiving rachitic rations fortified with cod liver oil and stored and with fresh cod liver oil.

FIG. 1. Chick No. 5908 from control group. Wide rachitic proliferating cartilage.

FIG. 2. Chick No. 5915. 0.5 per cent cod liver oil in ration. Stored 6 months.

FIG. 3. Chick No. 5933. 0.5 per cent cod liver oil. Freshly mixed in ration.

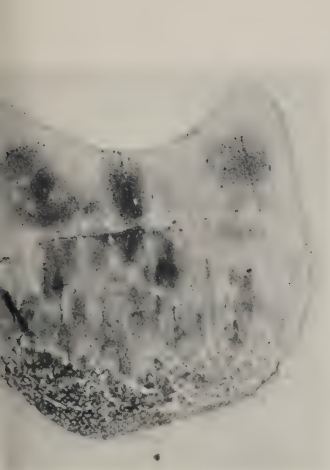


FIG. 1.



FIG. 2.



FIG. 3.

(Hart, Steenbock, and Lepkovsky: Antirachitic factor of cod liver oil.)

THE NUTRITIONAL REQUIREMENT OF THE CHICKEN.

V. THE INFLUENCE OF ULTRA-VIOLET LIGHT ON THE PRODUCTION, HATCHABILITY, AND FERTILITY OF THE EGG.*

BY E. B. HART, H. STEENBOCK, S. LEPKOVSKY, AND S. W. F. KLETZIEN,

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

AND J. G. HALPIN AND O. N. JOHNSON.

(From the Department of Poultry Husbandry, University of Wisconsin, Madison.)

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It has already been pointed out that the baby chick is extremely sensitive to rickets (1) and that the egg itself possesses distinct antirachitic properties (2). With the development of our knowledge of the relation of cod liver oil and light to a rachitic condition in the growing baby chick, it was but logical to inquire into the relation of ultra-violet light to egg production and particularly into the hatchability of eggs produced by hens receiving variable amounts of the antirachitic vitamin.

It is known to every expert poultryman that the hatchability of winter-produced eggs is relatively low, probably averaging not over 30 to 40 per cent of the fertile incubated eggs. It was conceivable that eggs produced in the late fall, winter, and early spring would be poorly endowed with antirachitic properties and that a paucity of this factor in the egg could lead to a weakness in the developing embryo, with the attending difficulty in getting out of the shell—in other words to a nutritional abortion in the shell. This situation is not without parallel. At this Station it has been demonstrated again and again (3) that a ration low in lime and the antirachitic factor can lead to disturbed reproduction in cattle and swine with weak or dead offspring as the result.

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Preliminary work on this problem was started in the fall of 1923, but because of the occurrence of "roup" among the birds the data were not so extensive, clean-cut, and conclusive as desired. In the meantime Hughes, Payne, and Latshaw (4) of the Kansas Agricultural Experiment Station have studied this problem and have secured data in general harmony with the data presented in this paper.

EXPERIMENTAL. .

On January 1, 1925, four groups of pedigreed white leghorn hens, hatched in the spring of 1923 and consequently with 1 year of laying completed, were placed on identical rations. The hens were selected as carefully and as uniformly in reference to laying history and breeding as could be done. These groups of hens (twelve in a lot) were housed in pens of similar size and similar light in the attic of the University Poultry Building. There were windows in this attic, but these windows were always closed. Ordinary electric lights were available in each pen and were turned on during the day. At night the pens were dark. No direct outside light reached the pens. All of the floors were provided with pine shavings litter.

The ration fed consisted of the usual poultry ration fed laying winter stock at this Station. It is of exceedingly great complexity—probably more so than need be, but apparently of very low antirachitic properties. It consisted of 20 parts of yellow corn, 10 of wheat bran, 10 of wheat middlings, 10 of gluten feed, 5 of ground oats, 5 of oil meal, 10 of beef scraps, 3 of pearl grits (calcium carbonate), and 0.5 of common salt. This mixture was fed as a mash. Skimmed milk was allowed *ad libitum*. Oyster shells were always before the birds and germinated oats were also fed daily. A scratch mixture consisting of two-thirds of whole yellow corn and one-third of whole wheat was also allowed. Probably 50 per cent of the dry matter consumed came from the scratch feed.

A male bird (pure bred leghorn) was placed in each of the first three pens, but was not exposed to ultra-violet light. Each male was left in each pen 24 hours and then rotated to the next pen of this series. For example, the male which was placed in Pen I for the first 24 hours was placed in Pen II for the next 24 hours

and in Pen III for the next 24 hours, and the other two male birds were rotated in a similar manner.

In Pen IV the male bird alone was subjected to ultra-violet radiation, but the hens of this group received absolutely nothing in addition to the basal ration. In summary the treatments were as follows:

Pen I. Basal ration plus 10 minutes ultra-violet light per day. Male bird not irradiated.

Pen II. Basal ration only. Male bird not irradiated.

Pen III. Basal ration plus 5 parts of dried pork liver. Male bird not irradiated.

Pen IV. Basal ration only. Male bird irradiated 10 minutes daily with ultra-violet light.

The ultra-violet light used was produced by a Cooper Hewitt quartz lamp operated on a 110 alternating current circuit. The hens were irradiated as a group at a distance of approximately 30 inches. The male bird in Group IV received similar treatment in respect to ultra-violet light.

Dried pork liver, fed at a level of 5 per cent of the mash of the ration, was added to the ration of Pen III because in some earlier work at this Station on the influence of the ration on the hatchability of eggs, the observation had been made that dried pork liver improved the hatchability when the basal ration consisted of *white* corn and skimmed milk supplemented with a calcium salt and sodium chloride.

Egg Production.

On February 1, 1925, collection of the eggs was begun with each hen trap nested. In Table I is given a summary of the eggs produced by each group monthly including the months of February to June inclusive. A study of the table shows the very marked influence the ultra-violet light had had on the number of eggs produced. Two to three times as many eggs were laid under the influence of ultra-violet light as without it. Pen I (irradiated) produced 173 eggs in the month of February, 178 in March, 211 in April, 189 in May, and in June 151; while Pen II produced in February 73 eggs, in March 59, in April 54, in May 37, and in June 29.

After April 15 the continuation of Groups III and IV on their respective rations was abandoned. It became apparent that all groups not irradiated were beginning to suffer from rickets, even before this time. This condition was apparent from the frequent fluttering of the hens when trying to walk, coupled with repeated squatting. In some cases what the poultryman has called egg paralysis was present. This is a failure or inability of the hen to expel the egg from the oviduct, resulting in paralysis and death. This condition manifested itself in the birds not irradiated and began to show after 12 weeks of confinement. It was more prevalent in Pens III and IV than in Pen II. In fact, not

TABLE I.
Record of the Number of Eggs Produced.

Pen No.	Ration.	Feb.	Mar.	Apr.	Apr. 1-14.	Apr. 15-30.	May.	June.
I	Basal plus 10 min. ultra-violet light daily.	173	178	211			189	151
II	Basal.	73	59	54			37	29
III	" plus 5 per cent dried pork liver.	68	47		24	18*	89	72
IV	Basal. Cockerel only irradiated 10 min. daily.	69	45		20	59†	183	150

* Began supplementing the ration with cod liver oil.

† Began supplementing the ration with ultra-violet light.

a single bird was lost from Pen II up to the time that the change was made in the ration, namely April 15. On the other hand by April 10 two birds had been lost from Pen III and three birds from Pen IV—all apparently suffering from rickets. Like Pen II, not a single bird had been lost from the irradiated group Pen I.

Further, in support of our supposition that these hens were suffering from rickets inorganic phosphorus determinations were made in the blood. While we are aware that low inorganic phosphorus in the blood is not an absolute criterion of a rachitic condition, yet the data shown in Table II indicate very clearly that the blood of the irradiated hens was distinctly higher in inorganic

phosphorus than the blood of those not receiving ultra-violet light. This fact was used to confirm our belief that the birds not irradiated were suffering from rickets. Consequently on April 5 the ration of Pen III was supplemented with 5 per cent of the mash as cod liver oil and in the case of Pen IV ultra-violet irradiation of 10 minutes per day was initiated.

Egg production had practically reached the zero point in both Pens III and IV at the time of the change. Only eight hens survived in Pen IV and nine in Pen III. Within 8 days after applying the ultra-violet light, egg production in Pen IV began to increase markedly and during the last half of April (15 to 30) 9 eggs were laid by this group, while but 20 eggs were produced

TABLE II.
Record of Inorganic Phosphorus in Blood of Irradiated and Non-Irradiated Hens. Blood Taken April 4, 1925.

Hen No.	Treatment.	Serum.	Hen No.	Treatment.	Serum.
		<i>mg. per 100 cc.</i>			<i>mg. per 100 cc.</i>
118	Irradiated.	4.70	106	Non-irradiated.	2.80
119	"	4.70	107	"	3.20
120	"	4.70	109	"	3.30
121	"	5.00	110	"	4.20
122	"	6.90	111	"	3.70
123	"	4.50	112	"	3.90

in the first half of that month. In May 183 eggs were laid by the eight hens of Pen IV receiving the ultra-violet light since April 5, and in June 150 eggs.

The cod liver oil feeding gave a slower response, but, nevertheless, response was made. While this group of eight hens only produced 20 eggs during the last half of April (cod liver oil feeding as begun April 15), yet in May they produced 89 eggs and in June 72 eggs. These data show the distinct response to cod liver oil feeding although this response was not so immediate or so pronounced as with ultra-violet light radiation. This phase of the field—the study of the influence of feeding the antirachitic vitamin on egg production—needs more study. Whether the slower response through feeding was due to the quantitative

difference in the vitamin D content of cod liver oil as compared to the activating influence of the ultra-violet light or to the difficulty of absorption of vitamin D from foodstuffs cannot at present be stated.

TABLE III.
Record of the Hatchability and Fertility of Eggs.

Pen No.	Treatment	Month.	No. of eggs incubated.	No. of eggs fertile.	Fertility. per cent	No. of chicks hatched.	Total eggs hatched. per cent	Fertile eggs hatched. per cent
I	Irradiated.	Feb.	168	157	93	103	61	66
		Mar.	149	141	94	91	61	64
		Apr.	207	203	98	142	68	70
		May.	190	184	96	126	66	68
II	Non-irradiated.	Feb.	69	62	90	22	31	35
		Mar.	43	30	70	6	14	20
		Apr.	50	36	72	0	0	0
		May.	35	24	70	0	0	0
III	" plus 5 per cent pork liver.	Feb.	61	54	89	11	18	20
		Mar.	33	19	60	2	7	10
		To Apr. 15.	20	12	60	0	0	0
		Apr. 15-May 1.*	18	11	60	0	0	0
		May.	87	62	71	26	30	41
IV	Male only irradiated.	Feb.	66	59	89	26	39	44
		Mar.	31	26	84	3	9	11
		To Apr. 15.	17	15	88	1	6	6
		Apr. 15-May 1.†	59	50	84	31	52	62
		May.	187	164	89	125	66	76

* Began cod liver oil feeding.

† Began irradiation of hens.

Hatchability and Fertility of Eggs.

Hatching and fertility records of the eggs produced by the four groups are given in Table III. It will be seen that there was a marked increase in the number of eggs hatched from the irradiated pen as compared with the other three groups, but particu-

early Groups II and III. In the irradiated group hatchability was sustained and possibly improved as the experiment progressed. At the end of May, 5 months after initiation of the experiment, the hatchability of the eggs from the irradiated hens was 66 per cent as compared with 61 per cent in February. With the non-irradiated hens the hatchability of the eggs had gradually receded. In February it amounted to approximately 30 per cent in the non-irradiated group, but by the end of April it was impossible to get a single chick out of the shell. By this time hatchability was practically zero (see Chart 1).

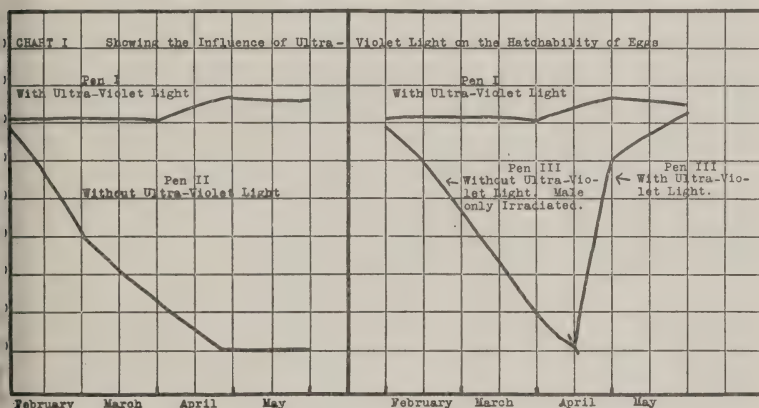


CHART 1.

The addition of dried pork liver to this ration had had no influence on the hatchability or fertility. Possibly the explanation of its apparent efficiency in the earlier experiments where the ration consisted of *white* corn and skimmed milk is to be related to a reinforcement of the ration with vitamin A.

The hatchability of the eggs from Pen IV where the male bird only was irradiated seemed to be somewhat improved, especially in the early part of the experimental period. Possibly this was due to the consumption by the hens of some of the excreta of the irradiated male or to the transfer of small amounts of antiradiationally active material with the seminal fluid.

Fertility was slightly better in Pen I than in the other lots,

but the differences were not sufficiently consistent to warrant further consideration at this time.

Calcium and Phosphorus Content of Eggs.

The fact that the hatchability was markedly improved by ultra-violet light led us to make determinations of calcium and phosphorus in the dried parts of the eggs from the irradiated and non-irradiated groups, with the possibility of more closely correlating low hatchability with low mineral content of certain parts of the egg and with a low antirachitic potency of the egg embryo (yolk). These data are presented in Tables IV and V.

It is to be seen from this record that the dried weight of the eggs produced by the irradiated hens was slightly greater than in the case of the non-irradiated groups. The whites were also slightly heavier. In the case of the yolks the differences were not great enough to merit particular attention. The dried weight of the shells were as a rule distinctly heavier in the case of the eggs produced by the irradiated hens. This is of particular significance as it indicates clearly the rôle of an abundant source of the antirachitic factor in egg shell building.

Further, the making of a heavier shell is of distinct practical significance particularly in the marketing of eggs. The more abundant supply of calcium carbonate in the shells of eggs from irradiated hens as compared with non-irradiated hens might also be of significance in reference to the supply of lime for the developing embryo. However, the amount of lime transferred from the shell to the developing embryo is such a small proportion of the total lime content of the shell (5 to 10 per cent) that the relation is probably of no great significance. The percentage of lime in the shells from the two groups was practically alike. The same statement can be made in reference to the per cent of lime in the dried whites and dried yolks. There seemed to be a tendency for the lime of the yolk of eggs from irradiated hens to run slightly higher in percentage than the percentage of lime in the dried yolks of non-irradiated hens' eggs. However, the difference was very slight.

The per cent of phosphorus in the dried whites and dried yolks of the two groups of eggs was not distinctly different. The average dry weights of the yolks and whites of the eggs from the

TABLE IV.
*Record of Calcium Content of Eggs from Irradiated and Non-Irradiated Hens
Taken 100 Days after Initiation of Experiment.*

Hen No.	Treatment.	Dry weight of egg shell.	CaO in dried shell.	Dry weight of egg white.	CaO in dried white.	Dry weight of yolk.	CaO in dried yolk	Dry weight of whole egg.
		gm.	per cent	gm.	per cent	gm.	per cent	gm.
18	Irradiated.	6.312	53.3	4.363	0.39	10.463	0.39	21.138
20	"	6.041	53.2	4.295	0.39	10.767	0.45	21.103
28	"	5.671	53.8	4.807	0.36	9.970	0.46	20.448
29	"	5.666	52.2	4.260	0.42	9.894	0.51	19.820
19	"	5.302	52.7	3.670	0.30	9.163	0.37	18.135
23	"	6.146	53.1	3.872	0.25	9.790	0.39	19.808
28	"	5.602	53.1	4.991	0.20	9.450	0.45	20.043
06	Non-irradiated.	5.275	52.8	3.524	0.37	9.249	0.36	18.048
13	"	3.268	51.0	3.487	0.42	8.482	0.31	15.237
14	"	3.190	48.8	3.885	0.36	10.663	0.42	17.738
17	"	4.508	52.7	3.265	0.42	8.956	0.40	16.729
15	"	3.640	51.5	2.924	0.30	8.156	0.29	14.720
10	"	4.020	51.1	3.663	0.31	9.487	0.38	17.170
06	"	4.909	52.3	3.487	0.32	8.674	0.42	17.067

TABLE V.
*Record of Phosphorus Content of Eggs from Irradiated and Non-Irradiated
Hens. Taken 100 Days after Initiation of Experiment.*

Hen No.	Treatment.	Dry weight of egg white.	P ₂ O ₅ in dried white.	Dry weight of yolk.	P ₂ O ₅ in dried yolk.
		gm.	per cent	gm.	per cent
118	Irradiated.	4.363	0.20	10.463	2.79
120	"	4.295	0.27	10.767	2.79
128	"	4.807	0.31	9.970	2.87
129	"	4.260	0.25	9.894	2.79
119	"	3.670	0.39	9.163	2.78
123	"	3.872	0.33	9.790	2.88
128	"	4.991	0.28	9.450	2.82
06	Non-irradiated.	3.524	0.28	9.249	2.78
13	"	3.487	0.24	8.482	2.68
14	"	3.885	0.32	10.663	2.69
17	"	3.265	0.27	8.956	2.66
15	"	2.924	0.42	8.156	2.64
10	"	3.663	0.34	9.487	2.71
06	"	3.487	0.32	8.674	2.75

irradiated hens were slightly higher than the dry weights of the yolks and whites from the non-irradiated groups, but not distinctly so. With approximately equal per cents of phosphorus in the two classes of eggs, the total phosphorus in the yolks and whites would be slightly higher for the eggs from the irradiated group; but the differences are too slight to be of significance and do not stand out in definite contrast as do the differences in the lime content of the shells. Constancy of composition of an essential living structure such as the embryo—whether in plant or animal life—has always been observed, while the greatest variations may occur in organs of storage.

Calcium Content of Embryo.

The improved *hatchability* of the eggs produced by irradiated birds is very probably related to the increased amounts of the antirachitic factor in the egg. These compounds, no doubt favorably influence the transfer of calcium from the shell to the developing embryo and thereby prevent the formation of a rachitic embryo, if one may speak of such a condition, during development. Confirmation of this supposition is seen in Table VI.

In securing the data shown in this table chicks from the irradiated groups were taken after they had been out of the shell 1 to 2 hours. They were killed, cut open, and dried at 65°C. They were then cut up and extracted with hot 95 per cent alcohol for 3 days. After the extraction the residue was dried, ashed, and the calcium determined in the ash. From the non-irradiated hens the hatchability of the eggs had receded to such a low point that seldom did we get a living chick out of the shell. This was toward the latter part of April or approximately 4 months after the initiation of the experiment. However, the material used from the non-irradiated hens consisted only of those embryos that had been alive for 21 days; some of them had made an attempt to get out of the shell and pipped, while others had not. In some cases the yolk had not been drawn into the abdominal cavity while in other cases it had. These data confirm very strongly the point of view that the eggs of the non-irradiated hens were so low in their content of the antirachitic factor as to make impossible the optimum transfer of lime from the shell to the developing embryo. In fact, the amount of lime in the chick from irradi-

TABLE VI.
Record of Calcium Content of Chicks at Time of Hatching (21 Days) from Irradiated and Non-Irradiated Hens. Taken 100 to 120 Days after Initiation of Experiment.

Hen No.	Treatment of hen.	Weight of dried chick.	Weight of ash.	CaO.	Weight of CaO.	Hen No.	Treatment of hen.	Weight of dried chick.	Weight of ash.	CaO.	Weight of CaO.
		gm.	gm.	per cent	gm.			gm.	gm.	per cent	gm.
118	Irradiated.	7.030	0.532	39.0	0.207	150	Non-irradiated.	6.174	0.320	32.5	0.104
119	"	5.750	0.524	39.0	0.209	145	"	6.395	0.325	34.4	0.112
120	"	6.032	0.545	43.5	0.237	150	"	7.656	0.252	33.3	0.084
123	"	6.267	0.546	39.5	0.216	148	"	5.985	0.305	33.3	0.102
125	"	6.948	0.542	41.4	0.214	145	"	5.543	0.295	31.8	0.094
127	"	6.848	0.612	39.2	0.240	117	"	5.282	0.320	40.7	0.130
128	"	6.973	0.578	40.8	0.236	152	"	5.503	0.354	37.49	0.132
129	"	6.048	0.507	44.7	0.226	148	"	5.998	0.322	32.75	0.105
120	"	6.099	0.444	42.5	0.188	192	"	5.490	0.339	38.51	0.130
129	"	6.060	0.483	44.4	0.214	116	"	5.764	0.270	31.31	0.084

ated hens' eggs was approximately twice that in the chicks from the non-irradiated hens' eggs.

Antirachitic Properties of Egg Yolks from Irradiated and Non-Irradiated Hens.

From the poorer results secured on the hatchability of the eggs produced by the non-irradiated hens as contrasted with the irradiated group, we suspected that there would be a difference in the antirachitic properties of the two classes of eggs. In fact a distinct difference in such properties would help greatly in explaining the results secured on hatchability. Consequently the antirachitic properties of the egg yolks of these groups were determined.¹

The determinations were made with rats and by the use of the ration and technique followed in our laboratory and developed by Steenbock and Nelson (6). In its essentials this procedure consists of the induction of a rachitic condition in the growing young rat by the use of a ration relatively rich in calcium as compared to its phosphorus content. When the rats showed evidence of a rachitic condition the egg yolk from the irradiated and non-irradiated hens' eggs was incorporated into the ration at varying levels.

The experimental material represented a mixture of the yolk of a number of eggs from each group.

The eggs used were laid March 18, 19, and 20; they were kept in a cool place until ready for use, and the yolks then fed to the rachitic rats for a 10 day period. At the end of this time, the rats were killed, the radii removed, and the silver nitrate test applied. In preparing the yolks, all of the eggs from a particular pen were taken, broken individually into a dish, and the yolk removed from the dish with as little adhering white as possible. All the yolks from a particular pen were then placed in a beaker, an equal weight of distilled water added, and the mixture thoroughly stirred. Into 100 gm. of the ration there were then introduced (on an undiluted basis) 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 gm. c

¹ These data are taken from the work of Professor J. E. Dougherty of the University of California, collected while in residence as a graduate student at the University of Wisconsin. They represent a part of the results of a more extended study of the problem which will be published later.

the egg yolk. After mixing the egg yolks with the ration the mixture was dried at 65°C. for 1½ days and then ground in a mortar to insure complete mixing. The results secured are tabulated in Table VII.

TABLE VII.

Record of Antirachitic Properties of Egg Yolks from Irradiated and Non-Irradiated Hens As Tested with Rats.

Irradiated hens.									
Trial I.					Trial II.				
Yolk added.	Sex.	Weight of rat when changed to yolk.	Final weight.	Line test.	Yolk added.	Sex.	Weight of rat when changed to yolk.	Final weight.	Line test.
gm.		gm.	gm.		gm.		gm.	gm.	
0.10	♀	86	95	—	0.10	♂	114	118	—
0.20	♂	101	111	—	0.20	♂	122	140	+++
0.50	♀	74	84	+++	0.50	♂	118	125	+++
1.00	♂	99	102	+++	1.00	♀	95	102	++++
2.00	♀	89	102	++++	2.00	♀	89	97	++++
5.00	♀	83	102	+++++	5.00	♂	110	132	+++++
Non-irradiated hens.									
Trial I.					Trial II.				
0.10	♂	98	102	—	0.10	♂	89	84	—
0.20	♂	112	118	—	0.20	♀	80	90	—
0.50	♂	98	104	—	0.50	♀	91	87	—
1.00	♀	93	93	—	1.00	♂	96	94	—
2.00	♀	99	92	++++	2.00	♀	84	92	—
5.00	♂	109	112	+++++	5.00	♂	89	97	++++

— equals no line; +, very narrow line; +++, wide line; +++++, very wide line; ++++++, complete healing.

It is very evident from the data secured that the yolks of the eggs from the irradiated group of hens were very much more antirachitically potent than those from the non-irradiated group. The ration fortified with 0.2 to 0.5 gm. of the egg yolks from irradiated hens was quite as potent antirachitically as the ration fortified with 5.0 gm. of the yolks from the non-irradiated hens. In other words, the yolks from the eggs of the irradiated hens were about

ten-fold as potent in calcifying properties as those from the non-irradiated hens.

Influence of Irradiation of Male Bird Only.

Irradiation of the male bird only (Pen IV) did not increase the production of eggs but the data indicate a slight improvement in the hatchability of the eggs from this group. Our limited data do not allow us to assert this as an established fact. If it is a fact it could be explained on the assumption that the hens kept in constant contact with the male consumed, through picking up of scratch feed, some of the excreta of the male which may have contained some of the antirachitic factor. This was found by Nelson and Steenbock (7) to be the explanation of the normal behavior of non-irradiated rats consuming rachitic diets when living in the same cage with irradiated rats. Whether the transfer with the seminal fluid of the cockerel of antirachitically activated material could also influence the hatchability of the egg is, of course, an undetermined point.

Can Direct Irradiation of the Egg Improve Its Hatchability?

While the facts detailed above show that the exposure of the bird itself to ultra-violet radiation will improve markedly the production and hatchability of eggs, it also was decided to determine if any such improvement could be accomplished through irradiation of the eggs directly. We were led to such an experiment because the successful antirachitic activation of a variety of foodstuffs when exposed in thin layers to ultra-violet light had been accomplished in this laboratory by Steenbock and collaborators (5, 6). We did not anticipate success in this experiment because of the probable failure of the ultra-violet light to penetrate the shell or albuminous envelope of the yolk. Groups of eggs in lots of 50 each were exposed to the radiations of a quartz mercury vapor lamp in periods varying from 1 to 40 minutes. In addition, groups of eggs unexposed to ultra-violet light were incubated with those that had been exposed. The results were as anticipated. Direct exposure to ultra-violet light had had *no influence* whatever on the hatchability of the eggs, nor had it killed the living embryo. The simplest explanation for these results is that the ultra-violet light had not penetrated the egg.

DISCUSSION.

It is clearly evident from the data available that light of certain wave-length has a very potent influence on the number of eggs produced as well as the hatchability of the egg of the chicken. In the months of February, March, and April, the group of hens receiving ultra-violet light produced 562 eggs; while those receiving no ultra-violet radiation produced 186 eggs in Pen II; 139 eggs in Pen III (to April 14); and 134 eggs in Pen IV (to April 14). It is a common experience that egg production is at a low ebb in the winter months, particularly for 2nd year hens, and gradually rises with the approach of the summer months and as the hens get out of doors in more sunlight. This fact, no doubt, is now to be correlated with the qualitative difference between winter and summer solar radiation in respect to its content of the rays of shorter wave lengths. With the advent of the fall and winter months and the gradual reduction in the quantity of ultra-violet light in solar radiation, there is a decreasing reserve of the antirachitic factor in the hen's body with an accompanying diminution in the number of eggs produced as well as the hatchability of these eggs. With access to ultra-violet light more of the antirachitic factor is produced; the assimilation of calcium and phosphorus is increased and a lesser number of deaths occur in the shell. As pointed out by Buckner, Martin, and Peter (8) restriction of calcium in the diet of the laying hen will lead to decreased hatchability of the egg. But it is evident from our results as well as those of Hughes, Payne, and Latshaw that a plentiful supply of lime in the diet, when there is a paucity of the antirachitic factor, will not favorably influence egg production and hatchability for a long time.

The shells of the eggs of the irradiated birds were on the whole distinctly heavier and contained more total calcium than those not receiving ultra-violet light. Often the eggs from the non-irradiated groups could not be removed from the nests without collapse. The shells were thin and paper-like. The eggs from some individuals showed this extreme condition more pronouncedly than others. Of course, shipping such eggs would be it of the question.

Whether decreased content of the antirachitic factor in the

hen's body as the result of feeding diets low in antirachitic properties or as the result of a paucity of ultra-violet light is to be held responsible for lowered egg production is certainly an interesting question for speculation. Apparently this is not the primary factor *per se* involved in the lowered production. The fact that the eggs differed in their antirachitic properties—those from the irradiated group being distinctly more potent antirachitically than those from the non-irradiated groups—makes it clear that the quantity of antirachitic vitamin available for the eggs produced is not the determining factor in the number of eggs laid. In other words, a standard or uniform percentage of the antirachitic factor in the egg is not a condition for its production. Apparently, then, an increase in egg production incident to a greater supply of the antirachitic factor—light—must be explained in some other way. Up to the present time no adequate and complete food consumption records have been made for the birds receiving the different treatments, but it is entirely conceivable that the differences in egg production relate to an improved efficiency in the mechanism of absorption not only of the inorganic, such as calcium and phosphorus, but also in respect to organic food structures common to the egg. The effect of the ultra-violet light is to restore to a normal condition an animal suffering from mild to severe rickets or osteoporosis.

SUMMARY.

1. The egg production of hens is greatly influenced by the supply in the diet or environment of the antirachitic factor, even when there is plenty of lime in the ration. Groups of hens selected in January, 1925, continued to lay abundantly when given in addition to their ration an exposure to ultra-violet light of 10 minutes daily; those not receiving the ultra-violet light decreased their egg production practically to the zero point.

2. Feeding cold liver oil or irradiating hens with ultra-violet light after a long confinement to a rachitic ration with resultant decrease in egg production again stimulates and increases egg production.

3. Irradiation of hens with ultra-violet light greatly improves the hatchability of the eggs. Under the influence of this light hatchability was sustained at 60 to 70 per cent while in its absence the hatchability receded to zero.

4. Irradiation of hens with ultra-violet light increased markedly the amount of lime in the shell as compared with eggs from non-irradiated hens. This fact is of significance in the marketability of the egg. The calcium and phosphorus content of the whites and yolks of the two groups was not distinctly different.

5. The developing embryo from the eggs of irradiated hens contained, after 21 days of incubation, nearly twice as much lime as the embryo from non-irradiated hens' eggs. The low hatchability on the rachitic ration is probably related to the low power of the developing embryo to transfer lime from the shell.

6. The antirachitic potency of egg yolks from irradiated hens was approximately about ten times that of the egg yolks from non-irradiated hens.

7. Fertility of the eggs was not consistently influenced by a paucity or abundance of the antirachitic factor.

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ACID- AND BASE-FORMING ELEMENTS IN FOODS.*

By GUY W. CLARK.

(From the California Stomatological Research Group and the Division of Biochemistry and Pharmacology, University of California, Berkeley.)

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The data presented here represent the analyses of foods which were used during two extensive experiments in adult mineral metabolism.

While ash analyses do not indicate the combination of acids and bases in our foodstuffs they afford us information as to the amounts of the various elements ingested and are therefore necessary in a complete study of mineral metabolism.

The data presented in Table I are in the nature of a supplement to the material presented by Sherman (1), Forbes (2), and others, and presumably indicate the effects that different soils and climate might have upon the mineral content of the various foods. While there are some marked differences between the figures given in this paper (see rice, prunes) and those presented by Sherman (1) it is surprising to find such general agreement.

Methods.

Sampling.—Some of the foods were fed during a period of 28 weeks, others 10, some 8, etc., but in all cases composite food samples were made during the entire time the food was used. The amount for the composite was always a definite portion of the total weighed out for the experimental subjects. These samples were kept in glass containers (Mason jars) and where necessary were preserved by the use of small amounts of formaldehyde and refrigeration. These composites were then ground fine in a food chopper and, in most cases, dried at 105°C. in an electric oven. The dry material was finely ground and stored in glass-stoppered containers.

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598 Acid- and Base-Forming Elements in Food

Analyses.

Calcium and Magnesium.—The foods were ashed in platinum dishes in an electric muffle furnace at low heat. The white ash was then moistened with

TABLE I.
Mineral Constituents of Foods. Expressed in Mg. per 100 Gm. of Edible Portion.*

Foods.	Year sampled.	Ca	Mg	Na	K	Cl	P	S	N
Bread, white	1923	14	12	447	103	582	86	95	1429
“ Graham	1924	29	37	321	151	628	135	102	1245
Crackers, Graham	1923	22	48	400	190	410	125	137	1412
	1924	27	43	520	335	648	117	103	1050
Shredded wheat (California)	1923	37	37	150	320	27	363	157	1763
	1924	37	133	120	345	83	416	150	1680
Rice, California polished . . .	1923	4	11	40	95	38	94	65	976
	1924	6	24		88	27	98	84	930
Milk, whole*	1923	113	11	65	138	102	95	31	486
	1924	117	12	55	123	114	98	34	500
Cheese, California full cream	1923	543	49	550	170	917	490	168	3976
	1924	728	74	535	270	1107	566	189	3890
Roast beef, canned (Libby and Armour)	1923	18	19	554	207	839	157	195	3489
Eggs	1923	43	11	12	123	155	192	158	7767
Peas, fresh green†	1923	25	39	50	315	47	115	65	1160
String beans, fresh green† . .	1923	68	22	96	314	50	72	39	511
Potatoes, peeled, raw†	1923	4	22	33	282	44	45	26	319
	1924	7	27	31	294	31	49	35	340
Tomatoes, California, solid pack	1923	5	12	107	232	188	19	24	166
	1924	7	12	22	206	40	21	14	150
Raisins, California seeded . .	1923	47	41	30	530	48	101	42	374
	1924	54	46	185	338	99	104	45	400
Dried apples	1923	14	12	235	403	61	52	21	180
Dried prunes, Santa Clara‡ .	1923	63	39	123	685	17	94	36	440
Jelly, apple base	1924	12	10	10	117	18	4		

* The values given for milk are expressed in mg. per 100 cc.

† These vegetables were sampled before cooking.

‡ It should be borne in mind that prunes contain an organic acid (quinic acid) which is not oxidized in the body. See papers by Blatherwick (4).

concentrated hydrochloric acid and approximately 25 cc. of hot water. Where silica was known to be present, in vegetables, fruits, cereals, etc., the solutions were evaporated to dryness on a steam bath and the residues dried at 105°C. until the odor of hydrochloric acid was no longer perceptible.

The residue was again thoroughly moistened with hydrochloric acid and after adding hot water the mixture was allowed to digest on a hot-plate for 10 minutes. The solutions were then filtered and made up to 100 cc. The volume of the aliquots taken for analysis depended upon the amount of Calcium and Magnesium present. An excess of oxalate ions was added to the hot solution (a volume of approximately 100 cc.) and the solution slowly neutralized with ammonium hydroxide to pH 5.6 to 5.8, using methyl red as an indicator. The solution was kept just below the boiling point for 20 minutes, allowed to cool, and filtered. The precipitate after being washed oxalate-free was dissolved in normal sulfuric acid, heated to 75°, and titrated with 0.05 N potassium permanganate. The filtrates from the Calcium determinations were treated in the customary way and the magnesium was determined as $Mg_2P_2O_7$. (It should be noted here that iron was not removed and this together with traces of silica gives values for Mg which are a trifle high.)

Sodium and Potassium.—Aliquots of the same solution as used for Calcium and Magnesium were employed. After evaporating to dryness to remove the excess acid, the residue was dissolved in 1 or 2 drops of HCl and a few cc. of hot water. To this solution was added 0.5 cc. of 5 per cent ammonium phosphate and subsequently powdered $Ba(OH)_2$ until the mixture was alkaline to litmus. After warming several minutes the solution was filtered and washed. After evaporating the washings to a small volume a few drops of saturated ammonium oxalate, a drop or two of NH_4OH ,¹ and an excess of powdered ammonium carbonate were added. This mixture was warmed several minutes, allowed to cool, filtered, and washed thoroughly, and the filtrate evaporated to a small volume. 1 cc. of 50 per cent ammonium sulfate was now added and the solution heated on a hot-plate for several minutes. It was then filtered and the filtrate received in a tared platinum dish. After evaporating to dryness the residue was dried in an air bath at 110°C. and then ignited to dull red to expel ammonium salts. When cool the residue was weighed as combined sodium and potassium sulfates. After dissolving in a small amount of water a few drops of dilute (1-4) hydrochloric acid and an excess of chloroplatinic acid were added. The potassium chloroplatinate, retained on a carefully prepared Gooch crucible, was thoroughly washed with ammonium chloride which had been saturated with potassium chloroplatinate.

Chlorine.—After weighing out food samples, 10 cc. of 10 per cent sodium carbonate and enough distilled water to wet the mixture thoroughly were added. After stirring with a glass rod the mixture was dried and subsequently ashed in the muffle furnace. The ash was covered with water and concentrated nitric acid added, being careful to prevent mechanical losses. After making up to definite volume an aliquot was titrated with 0.03 N silver nitrate (1 cc. = 1 mg. of Cl) according to the Volhard method.

Phosphorus.—If the food was known to have an excess of acid-forming

¹ For this determination it is advisable to prepare ammonium hydroxide from conductivity water and NH_3 gas.

600 Acid- and Base-Forming Elements in Food

elements the substance was thoroughly moistened with 10 per cent sodium carbonate and treated as described under chlorine. We have found it necessary to remove silica in many of the foods (fruits, cereals, vegetables), and two dehydrations are advisable. Phosphorus was determined by titration of the yellow ammonium phosphomolybdate and since silica forms a similar precipitate (ammonium silicomolybdate) it is necessary to remove it as completely as possible.

Sulfur.—After experiencing considerable difficulty with the sodium peroxide fusion the wet digestion method as proposed by Halverson (3) was selected. This method was checked by oxidation in an Atwater bomb calorimeter and with foods containing little or no silica the two methods agreed very closely. With material such as feces the Halverson method gave results 5 per cent higher than those obtained by oxidation in the bomb.

Space does not permit individual mention but the writer wishes to express full appreciation of the careful work of the several collaborators.

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A FURTHER INVESTIGATION OF THE CHEMICAL PROPERTIES OF INSULIN.

By D. A. SCOTT.

*From the Connaught Laboratories and the Department of Biochemistry,
University of Toronto, Toronto, Canada.)*

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In a recent paper (1) the writer showed that the primary action when trypsin was added to insulin was adsorption and that this was followed by proteoclastic destruction of the insulin. In the present study an attempt has been made to ascertain some of the chemical groups which insulin contains. With this object in mind, material of known potency has been subjected to the action of various reagents. The resultant material has been tested by the standard procedure to find out whether the chemical groups affected by each reagent are necessary for the physiological action of insulin. The nitrogen distribution of a purified insulin product has been determined and a combustion analysis has been carried out on a sample of the same material.

Since the discovery by Banting and Best (2) of the pancreatic hormone, insulin, much work has been done in attempting to ascertain its chemical nature. The original investigators first recognized its protein-like properties when they observed that trypsin destroyed the activity of insulin. Best and Macleod (3) found that the insulin from the ox showed a distinct biuret reaction (protein) while the insulin from the skate showed no biuret reaction (non-protein). They also observed that insulin was readily absorbed by charcoal and kaolin. Dudley (4) found that both pepsin and trypsin destroyed insulin and concluded that it appeared to be a complex protein derivative. He also showed that his insulin which was purified by an entirely different method from the Toronto insulin, gave protein reactions and that its physiological activity was destroyed by alkali. Doisy, Somogyi, and Shaffer (5) found that insulin had an isoelectric point (pH 5.0) and the purified product gave characteristic reactions for proteins.

Independently the isoelectric point was discovered by Walden of Eli Lilly and Company (6). Widmark (7) attempted to purify insulin by separating the active material from inert matter by the use of various organic solvents. He was not successful in dialyzing insulin. He suggests that insulin is an albumose. Piper, Allen, and Murlin (8) state that insulin is not a protein. They did not obtain any of the protein reactions in a purified insulin product prepared from the ox pancreas. Kimball and Murlin (9) report the preparation of a product which gives no protein reaction and which has a nitrogen content of from 4 to 6 per cent of its dry weight. Shonle and Waldo (10) conclude that the pancreatic substance containing insulin appears to be a proteose which gives typical protein reactions, and that further research is necessary to determine whether the active principle is a proteose or is merely associated with the protein fraction.

The explanation of the failure of (1) Best and Macleod and (2) Murlin, to obtain positive reactions for protein may be due to the fact that these investigators were dealing with very dilute solutions of insulin and that the dilution was beyond the accuracy of the biuret test. This is also the view of Somogyi, Doisy, and Shaffer (11) who first suggested this possibility.

The chemical work on insulin appears to indicate clearly that the active substance is closely related to a protein. If insulin is a protein it would be expected to have amino groups. Amino groups react with certain chemicals, *i.e.* formaldehyde, nitrous acid, etc. Thus it was hoped that by subjecting insulin to the action of these various reagents its chemical nature would be more clearly demonstrated. Further it was hoped that it might be possible to form an insulin compound with some reagent which would aid in the purification or identification of the active substance. It also seemed of interest to find out whether or not the amino groups were essential for the physiological action of insulin.

The insulin used in the following experiments is lot No. 405 and was prepared according to the method of Scott and Best (12).

Action of Formaldehyde.

Dodds and Dickens (13) have reported that formaldehyde up to a concentration of 20 per cent at ordinary temperatures, has

no effect on the physiological activity of insulin. Our experiments were carried out as follows:

5 cc. of formaldehyde solution were added to 20 cc. of 20 unit insulin (pH 2.5) and the temperature kept at 20°C. After 20 hours the formaldehyde was removed *in vacuo*. The resultant product was diluted and tested for potency on a series of test rabbits according to the method adopted by the testing department of the Insulin Committee (14). Other experiments were made on insulin solutions which were made alkaline with excess sodium bicarbonate. Control experiments were made on insulin solutions without formaldehyde which were made alkaline with sodium bicarbonate.

TABLE I.

Experiment.	Acidity	Time.	Temperature.	Loss in potency.
	pH	hrs.	°C.	per cent
20 cc. insulin + 5 cc. H ₂ O.....	2.5	20	20	0.0
20 " " + 5 " HCOH.....	2.5	20	20	30.0
20 " " + 5 " H ₂ O.....	2.5	20	40	0.0
20 " " + 5 " HCOH.....	2.5	20	40	40.0
20 " " + 5 " H ₂ O.....	2.5	20	60	0.0
20 " " + 5 " HCOH.....	2.5	20	60	60.0
20 " " + 5 " H ₂ O + NaHCO ₃ (excess).		1	20	0.0
20 " " + 5 " HCOH + NaHCO ₃ (excess).....		1	20	90.0

From Table I it will be seen that formaldehyde in acid solution decreases the physiological action of insulin. Increasing the temperature hastens the inactivation. In a solution made alkaline with sodium bicarbonate the insulin is inactivated almost immediately. If formaldehyde combines with an amino group, then there should be a decrease in the amount of amino nitrogen after the treatment of insulin with formaldehyde. Attempts to estimate any change in amino nitrogen by the Van Slyke micro amino nitrogen apparatus, however, were unsuccessful. This was probably due to the small amount of amino nitrogen in the insulin solution.

Attempts were made to activate the insulin made inactive with formaldehyde by (1) acidifying the solution (pH 1.5) and heating

at 80°C. for 30 minutes, (2) making the solution alkaline (pH 8.5). No increase in activity was obtained by either procedure.

Since aldehydes are good reducing agents, it was thought that the action of formaldehyde on insulin might be one of reduction. To test this hypothesis samples of insulin were subjected to the action of other reducing agents such as nascent hydrogen, sulfur dioxide, and sodium bisulfide. These substances all destroy the activity of insulin and attempts to recover the potency either by acid hydrolysis or oxidation of the reduced insulin were unsuccessful.

Action of Benzoyl Chloride.

Primary and secondary amines, alcohols, and phenols yield benzoyl derivatives by the replacement of the hydrogen of the NH_2 , NH and OH groups by the benzoyl radicle. The formation of such derivatives not only indicates the presence of NH_2 , NH or OH groups, but also is used to identify the individual substances under examination, as the benzoyl derivatives crystallize well and have very definite melting points.

Experiments to demonstrate the action of benzoyl chloride on insulin were carried out as follows:

20 cc. (400 units) in insulin were made alkaline with sodium carbonate. To this were added 2 cc. of benzoyl chloride. The mixture was shaken for $\frac{1}{2}$ hour, acidified with hydrochloric acid, and the benzoic acid ethered out. The aqueous fraction after the removal of traces of ether *in vacuo* was tested for potency. No activity was present. Attempts were made to recover the active principle by acid hydrolysis, *e.g.* heating the solution for 30 minutes at 80°C. at pH 1.5. These attempts were unsuccessful (see Table II).

Action of Carbon Bisulphide.

Primary amines condense with carbon bisulfide with the formation of dialkylated thioureas. The latter are decomposed on boiling with concentrated hydrochloric acid yielding isothiocyanates (mustard oils). Kodama (15) has shown that certain amino acids condense with carbon bisulfide in the presence of sodium bicarbonate.

Experiments were carried on as follows:

100 cc. of insulin (1000 units) were made alkaline with sodium hydroxide (pH 9.5). To this were added 250 cc. of ethyl alcohol and 20 cc. of neutral carbon bisulfide. The mixture was shaken for 1 hour and was then acidified with hydrochloric acid. The carbon bisulfide and alcohol were removed *in vacuo*. A precipitate settled out during the concentration. This precipitate was soluble in a dilute alkaline aqueous solution. Samples of this solution were tested on standard test rabbits. A control experiment was carried out in which no carbon bisulfide was added. The above fractions were also tested for potency after an attempt had been made to regain the activity by acid hydrolysis. These tests showed that no potency was obtainable (see Table III).

TABLE II.

Experiment.	Amount injected.	Blood sugar.	
		Normal.	After 2 hrs.
	cc.	per cent	per cent
100 cc. solution from benzoyl chloride experiment.	1	0.110	0.104
	5	0.118	0.110
100 " " " " " after acid hydrolysis.	1	0.115	0.118
	5	0.104	0.120
Control experiment (benzoyl chloride and water).	1	0.118	0.110
	5	0.110	0.114

Action of Nitrous Acid.

Nitrous acid reacts with warm solutions of primary amines yielding alcohols, with secondary amines yielding nitrosamines. Nitrous acid was first used as a deaminizing agent for proteins in 1885 by Loew (16). This investigator found that one-third of the nitrogen of peptones was liberated by the action of nitrous acid.

Several procedures were used in an attempt to deaminize insulin. The first was the method of Levites (17). 50 cc. of 10 unit insulin were acidified with 5 cc. of glacial acetic acid. This solution was warmed to 40°C. in a water bath. To the solution were then added 10 cc. of a 10 per cent solution of sodium nitrite. This mixture was retained at a temperature of 40°C. and shaken at intervals over a period of 1 hour. A precipitate settled out. At the end of 1 hour the solution was cooled to 20°C. and made

alkaline (pH 8.5). At this pH the precipitate was completely soluble. Samples of the resultant product were tested on standard rabbits. Approximately 75 per cent of the activity was destroyed. Part of the solution was acidified (pH 1.5) and hydrolyzed. It was then made alkaline and tested for activity. No potency was recovered. It is possible that the heat at 40° C. may cause a slight hydrolysis or other changes in the insulin protein structure, although this is scarcely likely, as it has been shown that insulin is quite stable in acid solution at much higher temperatures. However, in order to test this possibility the method of Dunn and Lewis (18) was tried. 50 cc. of 10 unit

TABLE III.

Experiment.	Amount injected.	Blood sugar.	
		Before.	After 2 hrs.
	cc.	per cent	per cent
100 cc. insulin (pH 9.5) treated with CS ₂ and alcohol as described.	0.3	0.110	0.118
	2.0	0.110	0.098
	4.0	0.118	0.098
50 cc. of above sample hydrolyzed at pH 1.5.	2.0	0.114	0.104
	4.0	0.120	0.098
100 cc. insulin (pH 9.5) treated with alcohol (control experiment).	0.3	0.118	0.040
	0.4	0.110	Convulsions.

insulin were added to 5 cc. of glacial acetic acid. To this solution 10 cc. of a 10 per cent solution of sodium nitrite were added. The sodium nitrite was added dropwise and the solution was kept well shaken. A yellow precipitate gradually settled out. After standing 18 hours the solution was made alkaline (pH 8.5) and tested for activity. Attempts to recover the potency by acid hydrolysis were also made. Negative results were obtained. To determine whether or not the concentration of the reagents used in these experiments had any effect on blood sugar, experiments were carried out exactly the same as above with the exception that water was used in the place of insulin. Amounts of this solution equivalent to the amounts used in the first two experiments were injected into standard test rabbits. The results were negative (see Table IV).

Chemical Assay of Insulin.

This investigation is an attempt to obtain a better idea of the complexity of the insulin protein molecule. The first step in such an undertaking is the preparation of a pure product. After many efforts in which many different methods of separating proteins were tried, the following procedure was adopted for the preparation and purification of insulin. The procedure of preparation is similar to that previously reported by Scott and Best (12).

TABLE IV.

Experiment.	Acidity.	Amount injected.	Blood sugar.		Loss in potency.
			Normal.	After 2 hrs.	
	<i>pH</i>	<i>cc.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Method of Levites as described, volume = 100 cc.	8.5	3	0.110	0.048	Approximately 75
	2.5	3	0.118	0.040	
Method of Levites as described after acid hydrolysis.	8.5	3	0.120	0.054	
Method of Dunn and Lewis as described, volume = 100 cc.	8.5	3	0.115	0.051	Approximately 70
	2.5	3	0.118	0.054	
Method of Dunn and Lewis as described after acid hydrolysis.	8.5	3	0.118	0.048	
Control experiment in which water replaced the insulin solution.	8.5	3	0.104	0.110	Normal.

Fresh pancreatic glands from the ox were obtained from abattoirs. As much fat and connective tissue as possible were separated from the glands. They were then collected hourly and placed in a refrigerator at 4°C. The glands representing the morning's kill were collected and taken to the laboratory at 12 o'clock noon.

The glands were weighed. They were then run through a power meat chopper in which they were finely minced. The minced material dropped into large 50 gallon earthenware crocks. For every 100 lbs. of glands there were added 25 gallons of 95 per cent alcohol and 5 gallons of water containing 10 cc. of concentrated hydrochloric acid. (The alcohol was ethyl denatured with 10 per cent methyl alcohol.) The mixture was slowly agitated in order to facilitate the extraction. At the end of 3 hours the acid-alcohol mixture was poured into a large rotary centrifuge which separated the

alcoholic extract from the solid materials. The solid materials remaining in the centrifuge were reextracted with a volume of 60 per cent alcohol equal to that of the liquid obtained during the centrifuging. After this mixture had stood overnight, it was centrifuged as in the case of the first extraction. The extracts from the first and second extractions were mixed and made alkaline with ammonium hydroxide. The mixture was filtered through large glass funnels which had been fitted with fluted filter papers. The filtrate was then acidified with sulfuric acid. 125 cc. of concentrated sulfuric acid solution were added for each 50 gallons of filtrate. The alcoholic extract was then concentrated to about one-tenth of its original volume in an efficient vacuum still. During the distillation, the temperature of the distillate was not allowed to rise above 30°C.

After the completion of the distillation, the concentrate was quickly heated to 50°C. At this temperature, lipid and other materials were readily removed by filtration. The concentrate had an acidity of pH 2. It is important that the acidity be within this range (pH 2 to 2.5) in order to ensure rapid filtration and to avoid loss of potency. After the concentrate had cooled, ammonium sulfate was added to half saturation (37 gm per 100 cc.). This mixture was stirred, and almost immediately protein material separated out and readily rose to the top of the liquid. After standing overnight, the protein precipitate was skimmed off and as much liquid as possible was pressed out. The weight of the precipitate from 300 lbs. of glands was approximately 200 gm. This precipitate was dissolved in hot acid alcohol. 1 liter of 50 per cent alcohol containing 5 cc. of 5 N HCl was sufficient. The temperature was kept between 30° and 40°C. When the precipitate had completely dissolved ten volumes of warm alcohol were added. The solution was then neutralized by adding 5 cc. of 5 N NaOH. After the solution had cooled to room temperature it was placed in a refrigerator at 5° C. for 2 days. At the end of that time the dark colored supernatant alcohol was filtered through a hardened filter paper on a Büchner funnel. The alcohol contained practically no potency. The precipitate was washed with ether and dried. It was then dissolved in acid water in which it was readily soluble. The solution was made alkaline with 5 N NaOH to pH 7.5. At this alkalinity a dark colored precipitate settled out (see Shaffer *et al.*). This was immediately centrifuged off. This precipitate was washed once or twice with alkaline water, pH 9.0, and the washings added to the main liquid. It is very important that this process be carried out fairly quickly as insulin is destroyed in alkaline solution. The acidity was adjusted to pH 5 and a white precipitate readily separated out. After standing overnight in an ice chest the supernatant liquid was decanted off and the resultant liquid removed by centrifuging. The precipitate was then dissolved in a small quantity of acid water.

A concentrated solution of trichloroacetic acid was added to the acid insulin solution until a concentration of 3 per cent was reached. The insulin was completely precipitated by this concentration of trichloroacetic acid. Hiller and Van Slyke (19

have shown that trichloroacetic acid up to a concentration of 5 per cent is practically specific for precipitating protein. The more recent work of Wasteneys and Borsook (20) confirms the findings of these investigators. The protein precipitate was centrifuged off. The precipitate was dissolved in acid water and the pH adjusted to 6.2. This was allowed to stand overnight. During this time a small precipitate settled out. This precipitate was removed and the supernatant liquid adjusted to pH 3.7. A precipitate readily separated out and was centrifuged off. The supernatant liquid was then adjusted to pH 5.0. The precipitate which formed was centrifuged off. This precipitate was dis-

TABLE V.

Source of material.	Nitrogen per unit of insulin.
	<i>mg.</i>
Pancreas*.....	12.0
Alcohol filtrate.....	0.90
Concentrate.....	0.70
Ammonium sulfate precipitate	0.52
First isoelectric precipitate	0.022
Final product.....	0.006

* The beef pancreas from which the insulin was prepared yields a maximum of 3300 units per kg. by acid alcohol extraction as described above. Based on these figures, the nitrogen of the pancreas per unit of insulin is 2 mg.

dissolved in a small quantity of 50 per cent alcohol. The insulin was precipitated out by adding twenty volumes of acetone. After standing overnight the solid material was filtered off on a hardened filter paper. This material was transferred to a vacuum desiccator and dried over phosphorus pentoxide. The resultant product was snow-white.

Table V demonstrates the decrease in total nitrogen at different stages in the purification of the insulin product.

A small portion of the purified insulin powder was weighed and dissolved in acid water. This solution was thoroughly tested for potency by the standard procedure. The solution was then diluted to a concentration of 100 units per cc. Samples of this solution were tested with various reagents to determine the different chemical groups which it contained. The results of a series of tests are shown in Table VI.

Combustion Analysis.

Samples of the standard insulin powder were subjected to a combustion analysis. The nitrogen was determined by the ordinary Kjeldahl method. The carbon and hydrogen were determined by oxidizing a known weight of insulin powder to carbon dioxide and water. The carbon dioxide was absorbed in a Fleming tower and the water in a phosphorus pentoxide tube. The operation

TABLE VI.

Chemical tests.	Results.
Biuret (C O N H).....	Positive.
Ninhydrin (a amino and free carboxyl).....	"
Hopkins-Cole (tryptophane).....	" (faint).
Ehrlich (indole) (tryptophane).....	"
Millons (tyrosine)	"
Xanthoproteic (benzene radicle).....	"
Ehrlich (diazo).....	"
Folin and Looney (tryptophane).....	"
" " " (tyrosine).....	"
Pauly (iminazol).....	"
Organic sulfur (cystine).....	"
Molisch (carbohydrate).....	Negative.
Phosphorus.....	"
Orcein (pentose).....	"
Iron.....	"

TABLE VII.

Sample No.	Weight.	C	H	N	Ash.
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.2492	50.76	6.57	14.0	0.4
2	0.2714	50.03	6.66	14.0	0.4

was carried out in a silica tube heated in an electric furnace. The ash was determined by the difference in weight of the platinum boat before and after combustion. The results of the two experiments are shown in Table VII.

Distribution of Nitrogen in Insulin.

The Van Slyke (21) method of protein analysis was used. By this method of analysis the distribution of nitrogen in the following

roups was determined: amide, humin, arginine, histidine, lysine, ystine, and the amino and non-amino nitrogen in the filtrate om the phosphotungstic acid.

Tryptophane and tyrosine were determined on 0.8 gm. of insulin owder according to the method of Folin and Looney (22). The verage results of four estimations are recorded in Table VIII.

Amino nitrogen was determined by the Van Slyke micro amino itrogen apparatus. The amount of insulin used in each deter-

TABLE VIII.

Sample No.	Tyrosine.	Tryptophane.
	<i>per cent</i>	<i>per cent</i>
1	9.7	0.82
2	10.1	0.78
3	10.3	0.79
4	9.9	0.85
Average.....	10.0	0.81

TABLE IX.

Temperature = 22° C.; pressure = 750 mm.

Sample No.	Weight of sample.	Amino nitrogen.	Amino nitrogen per gm. insulin.	$\frac{\text{Free amino N.}}{\text{Total N.}}$
	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>per cent</i>
1	0.08	1.20	0.00834	6.0
2	0.08	1.25	0.00865	6.2

After the deaminization of the insulin solution, the resultant product s neutralized and tested for potency on a series of rabbits. More than 4 per cent of the potency disappeared.

amination was 0.08 gm. The insulin was dissolved in dilute acetic aid. This was sucked into the deaminizing chamber. The chamber was shaken for 30 minutes at about 400 revolutions pr minute. Dunn and Lewis (18) found that such a procedure s necessary to completely deaminize casein. The total nitrogen s determined on 0.2 gm. of insulin by the Kjeldahl method. e results are shown in Table IX.

The estimation of the different groups of amino acids were rried out on 3 gm. of the purified insulin powder. This powder s dissolved in 50 cc. of 20 per cent hydrochloric acid. The

solution was refluxed in a 300 cc. flask for 30 hours. Van Slyke (23) has shown that 24 hours boiling at 100°C. is usually sufficient to obtain complete hydrolysis of a protein. The procedure followed in determining the different groups was that described by Van Slyke. Duplicate analyses were carried out. The phosphotungstic acid which was used to precipitate the hexone bases was purified according to the method of Winterstein (24). Before analyzing the insulin samples of gelatin were processed in order to become familiar with the technique and to serve as a check on the reagents used. The product after hydrolysis showed no potency when tested on rabbits.

TABLE X.
Distribution of Nitrogen in Insulin.

	Total nitrogen.	
	1	2
	<i>per cent</i>	<i>per cent</i>
Ammonia.....	9.6	9.8
Humin.....	0.4	0.4
Argenine.....	10.0	10.0
Histidine.....	5.2	4.7
Lysine.....	4.8	5.4
Cystine.....	0.5	0.6
Total bases.....	20.5	20.5
“ filtrate.....	67.0	68.4
Amino nitrogen of filtrate.....	64.2	66.2
Non-amino nitrogen of filtrate.....	2.8	2.2
Total nitrogen recovered.....	97.5	99.1

DISCUSSION.

Insulin of known potency has been subjected to the action of various reagents. The resultant product has been tested on a series of standard rabbits and the change in the physiological action of the insulin determined. The experiments dealing with the action of benzoyl chloride and carbon bisulfide show that these reagents completely inactivate insulin in an alkaline solution. It was thought that the action might be merely one of absorption. Hence attempts have been made to free the insulin by acid hydrolysis. These experiments were unsuccessful. Insulin is only slightly acted on by formaldehyde in acid solutions at room tem

erature. At higher temperatures a greater loss in potency occurs. Almost complete inactivation of the insulin occurs, if the reaction proceeds in an alkaline solution. Attempts to reactivate the insulin by acid hydrolysis were unsuccessful. Since aldehydes are good reducing agents it was thought that the action might be one of reduction. Accordingly attempts were made to activate the insulin by oxidation. No activation occurred. Insulin is destroyed by other reducing agents such as nascent hydrogen, sulfur dioxide, and calcium bisulfite. In these experiments attempts were made to follow the decrease in amino nitrogen before and after the action of each reagent on insulin by the Van Slyke micro amino nitrogen apparatus. These experiments were unsuccessful, due probably to the fact that the amount of amino nitrogen in the solutions under test was too small.

The action of nitrous acid on insulin was studied. Levites and Dunn and Lewis studied the action of nitrous acid on proteins. Their methods when applied to insulin showed that there was a decided decrease in the activity of the insulin after the treatment with nitrous acid. In later experiments when the amount of free amino nitrogen was determined by the Van Slyke nitrogen apparatus, the mixture which remained showed practically no potency. The greater action of the nitrous acid on the insulin here is probably due to the greater concentration of the reagents and to the vigorous agitation. Attempts to activate the insulin were unsuccessful.

A very pure insulin product has been prepared. The essential steps in this preparation are (1) extraction with hydrochloric acid alcohol, (2) precipitation of the concentrate with ammonium sulfate, (3) dissolving the precipitate and reprecipitating in warm alcohol, (4) fractional precipitation at different acidities, (5) precipitation with trichloroacetic acid. The resultant product was practically snow-white and had a nitrogen content of 0.006 mg. per unit of activity. The physiological assay showed 25,000 units of insulin per gm. of powder. This assay was checked by the testing laboratory of the Insulin Committee. Many experiments have been made in an attempt to purify the insulin further. These results have been negative.

The chemical tests on the purified insulin solution containing 10 units per cc. show characteristic protein reactions. The

color reactions for tryptophane were faint. Phosphorus was not present. The Molisch and orcein tests were negative.

The results from the combustion analysis show that the values obtained for carbon and hydrogen are within the limits of those values obtained for a typical protein. The nitrogen figure is somewhat low. However, this nitrogen value was obtained by the Kjeldahl method of estimating nitrogen and may be somewhat lower than the true nitrogen value, since some substances do not give their total nitrogen by this method of estimation. Cruto (25) reports the results of his analysis of insulin to be C 47.73, H 7.27, N 14.53. While these results are in fair agreement with the author's, yet we would seem to be dealing with quite a different product. He reports that 0.8 mg. is the amount of insulin necessary to lower the blood sugar of a 2 kilo rabbit from normal to 0.45 per cent in 2 hours. 0.12 mg. of our product is necessary to give the same results or our product is 6.7 times as potent as his. Shonle and Waldo's (10) combustion analysis on a highly purified product shows a much lower hydrogen value and a slightly higher nitrogen value than the results reported here. The ash of their product was high.

The ratio of amino nitrogen to total nitrogen is similar to that obtained for a true protein.

The following points are of special significance in the distribution of nitrogen: (1) the high amide and low humin nitrogen (2) the low cystine value, (3) the high amino nitrogen in the filtrate, (4) the low non-amino nitrogen in the filtrate, (5) the high tyrosine and low tryptophane values. Certain values reported in the nitrogen distribution are in fair agreement with some values reported by Doisy and Weber (26) and by Shonle and Waldo (10). The cystine value of our insulin, however, is much lower. The cystine value for insulin was checked against the sulfur of the blood. This standard gave a value for cystine of 0.9 per cent of the total nitrogen. This value is in fair agreement with the figure obtained by the method of Van Slyke. Probably the high cystine values which other investigators have obtained are due to the fact that sulfuric acid was used in their method of preparing insulin whereas only hydrochloric acid was used in our method of preparation and purification.

From a consideration of the following points — (1) the active

of trypsin on insulin, (2) the action of specific reagents, (3) the preparation, (4) the chemical tests, (5) the combustion analysis, (6) the distribution of nitrogen—the similarity between insulin and a protein is apparent. Whether the purified product used in this study contains two or more proteins, having very similar physical properties, can only be determined by further research.

CONCLUSIONS.

1. Benzoyl chloride and carbon bisulfide completely inactivate insulin in an alkaline solution.

2. Formaldehyde and nitrous acid greatly decrease the activity of insulin.

3. Carbon, hydrogen, nitrogen, and ash values have been determined on a purified insulin product.

4. The distribution of nitrogen and the tyrosine, tryptophane, and amino nitrogen have been determined in a purified sample of insulin.

I wish to thank Professor Rogers of the Department of Chemistry for the carbon and hydrogen estimations and the Insulin Committee for checking the physiological assay. I am also indebted to Dr. C. H. Best and Dr. A. Hunter for their helpful criticism.

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UREA TESTS OF RENAL EFFICIENCY. I.

By I. M. RABINOWITCH.

WITH THE ASSISTANCE OF ALTHEA B. FRITH.

From the Department of Metabolism, Montreal General Hospital, Montreal, Canada.)

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The urea tests of renal efficiency commonly mentioned in the literature include (a) simple estimation of blood urea concentration, (b) determination of urine urea concentration following ingestion of urea, and (c) observations on the relationship between blood and urine urea concentrations. From a comparative study of these three methods, the general conclusion reached in this hospital (1) was that in renal lesions associated with azotemia or any other evidence of impairment in excretion of nitrogenous substances, the ratio of urine urea to blood urea concentration was the most sensitive index of renal efficiency.

All urea tests are based upon the clinical observation that when the kidneys become impaired the rate of excretion of urea diminishes. If the rate of excretion cannot keep pace with the rate of production urea accumulates in the blood. Rate of excretion of urea in the urine, and degree of retention of urea in the blood are therefore regarded as indices of kidney function. The assumption that these tests estimate the ability of the kidneys to do work connotes that excretory ability of the kidneys is the only, or at least predominant, factor influencing urea excretion. Ambard, Marshall, and Davis, Pepper and Austin, and Addis and his coworkers have recorded their observations on the effects of blood urea concentration. In 1921, Van Slyke and his coworkers demonstrated the influence of urine volume output (2). For normal individuals these authors presented a mathematical expression for the relationship of these factors. Thus, $K = \frac{D}{B\sqrt{VW}}$ where D represents gm. urea and V , volume urine output per 24 hours, W represents body weight (expressed in kilos), and B , blood urea concentration (gm. per liter). The value for K (called

the urea secretory constant) was found to be 7.5 ± 3 for normal individuals. Because of the wide variations noted in normal subjects, these authors concluded that blood urea concentration and urine volume output were not the only factors, aside from kidney excretory ability, governing the rate of urea excretion. For this reason they stated that their "urea secretory constant" must be regarded as "an object of investigation rather than an aid in the clinic." It might appear that the latter view would also apply to the "urea concentration factor." Especially so, since in the calculation of the latter no account is taken of urine volume output. The "urea concentration factor" represents merely the ratio of urine urea to blood urea concentration. Thus "factor"

$= \frac{U}{B}$, where U represents mg. urea per 100 cc. urine, and B represents mg. urea per 100 cc. blood. The latter has, however, in the great majority of instances yielded results consistent with the clinical picture, and has also been applied in a quantitative determination of the work done by the kidneys (kilogrammeter per gm. urea excreted), with fairly consistent results (3). It appears, therefore, necessary to attempt a logical explanation why the factor is a fairly accurate index of the excretory ability of the kidneys.

Since urine volume output and blood urea concentration affect urea excretion, it appears reasonable to assume that, if these variables be reduced to the minima, variations in the rate of urea excretion will be due chiefly to kidney excretory ability. The necessity of restricting fluids for 12 hours prior to the factor test, and discarding those tests in which a polyuric response is noted has been repeatedly emphasized (4). Under such conditions the volume output of urine rarely exceeds the rate corresponding to the minimum value of the "augmentation limit."¹ Austin, Stillman, and Van Slyke (2) have shown that the augmentation limit in normal subjects varied between 2.5 and 6 liters per 24 hour unit time. Also under the set of conditions for the test, the effect of blood urea concentration can hardly be great. It has been shown (1) that following ingestion of 15 gm. urea the mean increase in blood urea nitrogen was 9 mg. per 100 cc. blood. In a normal individual this would correspond, approximately, to a

¹ By the term "augmentation limit" is meant the maximum urine volume output necessary to produce the maximum rate of urea excretion.

per cent increase in blood urea concentration. The influence of such an increase may be judged from the data of Addis and Murray (5).

That these variables are controlled under the conditions of the test may be found in a comparative study of the "urea secretory constant" and the "urea concentration factor" obtained in the same individuals by the same procedure. Such study was made on twenty normal subjects and forty with albuminuria. The procedure of the test has been repeatedly described (1, 2, 4). In each case the values of $\frac{U}{B}$ and $\frac{D}{B\sqrt{VW}}$ were found. In the calculation of the latter the additional data necessary were urine volume and body weight. The normal and pathological cases were separately grouped.

TABLE I.

Group No.	$\frac{U}{B}$	$\frac{D}{B\sqrt{VW}}$ (mean value)
1	41 to 50	6.3
2	31 " 40	4.5
3	21 " 30	3.1
4	11 " 20	2.7
5	1 " 10	1.1

A statistical study was made of the normal group. The arithmetical mean, the standard deviation about the true value, and the coefficient of variation were calculated. The forty cases of albuminuria were divided into five groups according to the factor. Thus, Group 1 consisted of Factors 41 to 50, Group 2 consisted of 31 to 40, etc. In each group the value of the mean of the corresponding "constants" was found.

The factors and their corresponding constants (mean value) of the different groups of cases of albuminuria are recorded in Table I. The individual results are graphically recorded in Chart 1. The continuous line represents the values of $\frac{U}{B}$ plotted from highest to lowest. The dots represent the corresponding values of $\frac{D}{B\sqrt{VW}}$.

The parallelism between the values for $\frac{U}{B}$ and $\frac{D}{B\sqrt{VW}}$ is noted.

In normal subjects when no urea was given the value of $\frac{D}{B\sqrt{VW}}$

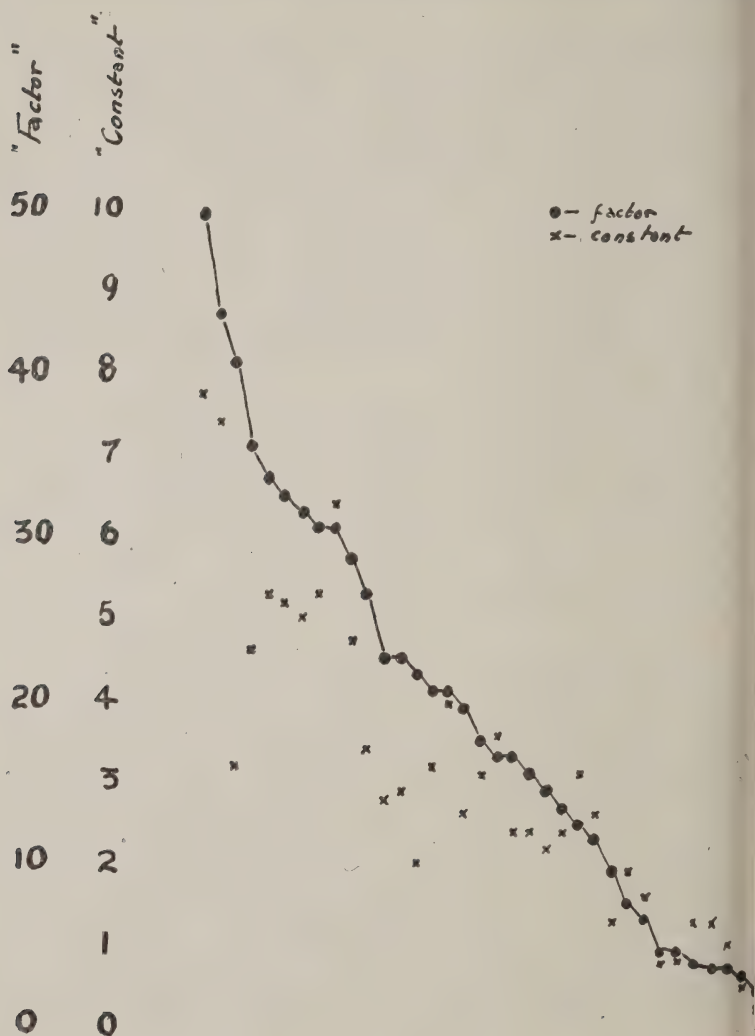


CHART 1. "Factor" values plotted from highest to lowest with corresponding values of "constant" in forty cases of albuminuria.

was 7.5 ± 3 (2). When urea was administered the value of the coefficient of variation was not only less, but was practically identical.

with that found for the factor, $\frac{U}{B}$. The mean value for the factor was 47.3 and the standard deviation about the true value was 6.9. The mean value for the constant was 9.8, and the standard deviation about the true value was 1.4.

TABLE II.

	Mean value.	Standard deviation.	Coefficient of variation.
When urea is given $\frac{U}{B}$	47.3	± 6.9	14.6
" " " $\frac{D}{D\sqrt{VW}}$	9.8	± 1.4	14.3
" no urea is given $\frac{D}{B\sqrt{VW}}$...	7.5	± 3	40

TABLE III.

Date.	Weight.	Blood urea-nitrogen.		Urine, 2nd hr. specimen.		$\frac{U}{B}$	$\frac{D}{B\sqrt{VW}}$	Remarks
		Before urea.	After urea.	Volume.	Urea.			
	kg.	mg. per 100 cc.	mg. per 100 cc.	cc.	per cent			
June 9	65.4	38	46	60	1.51	16.9	2.6	
" 14	65.7	34	43	55	1.44	17.4	2.5	
" 19	65.0	35	41	85	1.24	15.2	2.7	
July 4	64.7	28	42	105	1.60	21.3	4.2	No edema. Blood discs still present in urine.
" 15	64.0	24	31	95	0.94	14.2	3.0	" "
" 21	64.6	22	27	70	0.96	18.3	2.9	" "
" 28	64.6	21	37	95	2.76	44.4	8.3	No blood discs in urine.
Aug. 2	62.5	21	32	50	2.28	40.2	5.5	" "
" 8	61.8	22	35	45	2.76	45.2	6.0	" "

variation about the true value 1.4. Table II briefly tabulates the results. It would appear from the table that the administration of urea affects the variables. It is interesting here to note that Van Slyke² found that when urea is given there is less variation about the true value 1.4.

Personal communication.

tion in the relationship between blood urea concentration and urea excretion.

The following case is cited in illustration of (a) the parallelism between the factor and constant and (b) between the latter and the clinical picture.

Hospital No. 2155, male, age 27 years, was admitted to the Medical Service of Dr. A. H. Gordon, with a diagnosis of acute nephritis. The complete laboratory data are recorded in Table III.

A low factor and a low constant are noted during the acute stage of the illness. During the stage of convalescence they approach the normal values. Until July 21, though the patient appeared well for over 2 weeks previously, there was no alteration in the factor and in the constant. The only evidence of disease clinically was the persistence of isolated red blood cells in the urine. Following the disappearance of the blood from the urine both the factor and the constant increased in value. A parallelism between the clinical picture and factor, and between the factor and constant is thus noted.

RÉSUMÉ.

When urea was administered by mouth to normal individuals previously restricted from fluids for at least 12 hours, and tests showing polyuric responses, above the rate equal to the minimum value of the "augmentation limit" (2.5 liters per 24 hours), were discarded, the coefficient of variation in the case of the factor was practically identical with that of the constant. Under the same set of conditions a parallelism was also noted between the values of the factor and constant in forty cases of albuminuria.

Since urine volume output is essential in calculating the constant and is not taken into consideration in the calculation of the factor, and in view of the above observations, its effect on urea excretion is practically negligible under the set of conditions described. Excretory ability of the kidneys thus appears to be the predominant factor influencing the value of the ratio $\frac{U}{B}$. The ratio has, therefore, a sound basis as a test of renal efficiency.

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THE ISOLATION FROM BLOOD OF A HITHERTO UNKNOWN SUBSTANCE, AND ITS BEARING ON PRESENT METHODS FOR THE ESTIMATION OF URIC ACID.

BY GEORGE HUNTER AND BLYTHE A. EAGLES.

From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)

PLATE 2.

(Received for publication, July 17, 1925.)

In a recent paper on "Uric acid determinations in blood" it was shown by Bulmer, Eagles, and Hunter (4) that reliable values for uric acid could not be obtained by the addition of the uric acid reagents directly to Folin and Wu blood filtrates, a procedure first recommended by Benedict (1) and now extensively used initially for uric acid determination. It was shown that when the so called "direct" method was applied to Folin and Wu filtrates from certain animal bloods in which it is well known that the amount of uric acid does not exceed about 0.1 mg. per 100 cc. values were regularly found ranging from about 1.0 to 4.0 mg. The substance or substances responsible for the reduction of the molybdophosphotungstic acid in presence of sodium cyanide in animal bloods was found to be confined almost entirely to the corpuscles. Now it is well known that the direct method as recommended by Benedict for the estimation of uric acid gives values per 100 cc. of human whole blood on an average about 0.5 mg. higher than the figures obtained by the Folin and Wu (9) procedure by preliminary precipitation with silver lactate. Bulmer, Eagles, and Hunter failed to find any evidence for the commonly accepted belief that the extra 0.5 mg. given by the direct method was due to loss incurred by the extra manipulation required by the indirect method, and assumed the presence of an interfering substance which is separable from uric acid by the Folin and Wu silver procedure. Further justification for this

assumption was obtained from the fact that greater discrepancies were observed between the direct and indirect methods when using corpuscles than when using whole blood, and also from differences in the ratio of plasma : corpuscle uric acid obtained by the two methods on the same bloods.

The present paper marks a considerable advance on the findings of Bulmer, Eagles, and Hunter. Two non-protein substances have been isolated in the pure state from pig's blood, both of which give a blue colour with the uric acid reagents. From the point of view of the estimation of uric acid in human blood by the direct or indirect methods one of these substances is probably of little significance, and for present purposes will be dismissed from consideration. The other substance, as far as we can determine, has not hitherto been described. Conclusive proof of its structural formula may yet take considerable time, so we deem it desirable, especially from its importance in blood analyses, to report our chemical findings up to date. As the substance is relatively simple in structure we have refrained from suggesting a name for it until its chemical groupings have been determined. It will be referred to, as in the paper of Bulmer, Eagles, and Hunter, as "substance X."

The present paper is divided into two parts. Part I is a study of the bearing of the new substance on uric acid estimation in blood, and Part II a description of the method of isolation and properties of the pure substance.

PART I.

We were led to the isolation of substance X through some recent French findings which appear to have entirely escaped the notice of workers on this continent. As early as 1920, that is before the introduction of the direct method of Benedict, Grigaut (10) reported values obtained by a direct method from plasma using phosphotungstic acid along with sodium carbonate. In a paper reported in 1921 Grigaut (12) gave the mean normal uric acid content of human serum as 4.5 to 5.0 mg., of whole blood 15.0 mg. and of corpuscles 20 mg. per 100 cc. The key to the situation was furnished early in 1922 by Guillaumin (13) in the following figures.

	Filtrat direct.	Argent.
Plasma milligr. d'acide urique par litre	58	53
Corpuscules " " " " " "	205	28

In other words, plasma metaphosphoric acid filtrate treated by the precipitation method of Folin and Wu gives approximately the same uric acid value as plasma filtrate treated directly with the uric acid reagents, whereas corpuscle filtrate similarly measured shows an astonishingly high value by the direct method. During 1922 seven papers on the subject appeared in *Comptes rendus* by Guillaumin (14), Weil and Guillaumin (18), and by Chauffard, Rodin, and Grigaut (5). There is nothing of special significance for our present purposes in these papers which are for the most part of clinical interest and concerned with methods of deproteinization. As we shall show later the French results are not quite accurate, and in view of our findings will require to be revised. Yet the main findings of the French workers are of signal importance, although again their interpretation of the high values is unsatisfactory. Grigaut (11) examined a wide variety of substances amongst which alloxan and alloxantin were the only substances found which gave a colour with the Folin and Denis reagents, and thus concluded that only uric acid or its oxidation products alloxan or alloxantin could be responsible for the extra blue colour in corpuscles, although he leaves open the possibility that such a substance as that isolated by Davis and Benedict (6) might be contributing to the colour. But without commitment he prefers to speak of "*l'acide urique total*." Guillaumin has no further suggestions beyond dividing the substances responsible for the blue colour into "*l'acide urique libre*," and "*l'acide urique combiné*" and refers to the sum as "*l'acide urique total*" as does Grigaut.

We were entirely at a loss to explain the French figures but saw at once their parallelism with the findings of Bulmer, Eagles, and Hunter. It is well known that the use of sodium cyanide instead of sodium carbonate along with the phosphotungstic acid of Folin and Wu or with the arsenophosphotungstic acid of Benedict, with the aid of heat, increases very considerably the sensitivity of uric acid. We supposed that this increased sensitivity

with sodium cyanide was not shared by substance X, and that the presence of the latter would be made more conspicuous by sodium carbonate as used by the French workers. Later work has shown this assumption to be right, and has further shown why the direct method of Benedict for the estimation of uric acid in human blood has just failed to be a success.

EXPERIMENTAL.

Guillaumin (13) had shown that high uric acid values were obtainable from tungstic acid filtrates of corpuscles as well as from metaphosphoric acid filtrates. As a rule he obtained higher values using the latter, but as far as our experience goes there is little difference in the values, and, as clear metaphosphoric acid filtrates from corpuscles are very difficult to obtain, we have confined our attention solely to Folin and Wu filtrates.

Methods Used.—Three colorimetric procedures were employed in the course of the investigation of blood filtrates. Two of these have been described in the paper by Bulmer, Eagles, and Hunter (4) and were there referred to as the "direct" and the "indirect" methods. These arbitrary terms will be retained in this study. The direct method is that in which arsenophosphotungstic acid is used with sodium cyanide according to Benedict, on untreated filtrates; the indirect method that in which the blood filtrate is precipitated with silver lactate before addition of the uric acid reagents, according to Folin and Wu. The third method will here be referred to as the "French" method and is as follows for whole blood: 5 or 2.5 cc. of Folin and Wu blood filtrate are put in a short wide test-tube along with 6 or 8.5 cc. of water. 0.5 cc. of phosphotungstic acid, molybdenum free, according to Folin and Trimble (8), is next added, followed by 1.5 cc. of saturated anhydrous sodium carbonate (14.8 per cent), making a total volume of 13 cc. The blue colour developed is compared, after standing 5 minutes at room temperature, with a uric acid standard made at the same time from the Benedict and Hitchcock (3) standard containing 0.1 mg. of uric acid in 5 cc. 5 cc. of this solution are added to a test-tube similar to that used for the test solution, followed by 17 cc. of water. To this are added 1 cc. of the phosphotungstic acid reagent and 3 cc. of the saturated sodium carbonate, making a total volume of 26 cc. The standard thus contains 0.05 mg. of uric acid in 13 cc. In our earlier work we frequently used a standard of double this strength of uric acid but later have used such an amount of filtrate as will give a colour of approximately the same intensity as the 0.05 mg. standard. Corpuscle filtrates must be used similarly. The necessity for approximately the same intensity of test and standard colour will be seen later.

Table I shows some values obtained from human and other bloods, some of the values by all three methods, but all calculated on the basis of uric acid per 100 cc.

The results in Table I confirm the findings of the French workers on human blood. About 30 per cent discrepancy between the direct and indirect values for corpuscles is commonly met with, but the direct and indirect values for plasma are rather further

TABLE I.

Animal.	Corpuscles.	Calculated as uric acid per 100 cc.								
		Corpuscles.			Plasma.			Whole blood.		
		French.	Direct.	Indirect.	French.	Direct.	Indirect.	French.	Direct.	Indirect.
	<i>vol. per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Human.	46.6	16.8	2.81	1.79	3.42	3.12	2.58	9.64*	2.95	2.10
"	37.7	21.0	3.37	2.27	4.73	4.09	3.23	10.85*	3.48	2.91
"	46.5	18.8	3.26	2.22	4.36	3.66	3.21	11.07*	3.28	3.00
Dog 1.	40.0	14.3	2.45		1.2			6.0		
" 2.	35.4	24.0	5.26		0.9	1.46		8.0	3.03	
Rabbit 1.	22.0	88.9	12.0		2.0			19.6		
" 2.	31.0	31.5	6.7		2.1			11.3		
Sheep.		17.7								
Ox.		27.6			1.0					
Pig 1.		60.0	14.0							
" 2.		47.0								
Pigeon 1.		91.6	26.6	14.1	10.8	8.22	7.06			
" 2.		105.0	31.8	13.9	10.1	6.82	6.00			

* Calculated values.

part than is usually found in human bloods. As we have frequently observed, hemolysis accounts for this to some extent.

By the French method normal human blood commonly gives uric acid values from 8 to 15 mg. per 100 cc. and corpuscles about twice these values. In the same person's corpuscles we have found a change from 16.0 to 25.6 mg. in the course of a month.

By the French method average values for dog's blood are slightly lower than for human, while in rabbit they are considerably higher than human. Corpuscles of pig's blood nearly always contain more than 40 mg. per 100 cc.

A general parallelism is observed in the animal bloods in Table I between the values obtained by the French method and those obtained by the direct method. For the most part we have discontinued the indirect method on animal bloods, as the paper by Bulner, Eagles, and Hunter showed that with the exception of ox blood the indirect method showed inappreciable amounts of uric acid. By the direct method we find 2.45 and 5.26 mg. as uric acid, as compared with the corresponding values of 14.3 and 24.0 mg. respectively by the French method. In the two rabbit bloods there is a similar parallelism, whilst in pig's blood we find the very high value of 14.0 mg. as uric acid by the direct method. The obvious conclusion is that it is the same substance which is responsible for the production of blue colour with the uric acid reagents in both the direct and French methods. For human bloods the problem is more complex. Thus, it might be argued that if dog's blood showing a value of 14.3 mg. by the French method gives a direct value of 2.45 mg., then we should expect to find at least this difference between the direct and indirect methods in human bloods which show a French value of more than 14.3 mg. As the sequel will make plain, the presence of uric acid in human blood leads to a lower direct value than that obtained in the absence of uric acid.

It is necessary to state at this point that the values obtained by the French method are not a measure of substance X in the animal bloods, nor a measure of uric acid plus substance X in the human bloods. The other substance which we have isolated also contributes to this colour. There is the further consideration that substance X has a different sensitivity towards the French reagent than has uric acid (see below), and the other substance has yet a different sensitivity. For this reason we are at present reporting only a minimum number of values to confirm the French workers, and to make clear the parallelism between the values obtained by the French method and by the direct method.

Colour Values of the Pure Substance by the French Method.

It will be assumed at this stage of the discussion that substance X has been isolated in the pure state, in order that we may now consider its behaviour towards the uric acid reagents.

A solution in water of substance X was made containing 1 mg

per cc. From this requisite quantities were taken to measure the colour value of the substance as compared with that of uric acid by the French method and by the direct method.

The French method was done as described earlier for blood, water of course being added to make the volume 13 cc. Thus, when 0.5 mg. of substance X was used, 4.5 cc. of water were added and the procedure was continued as before. With amounts from 0.50 to 0.25 mg. of substance the colour was compared against a uric acid standard containing 0.1 mg. in 13 cc. From 0.20 to 0.05 mg. the uric acid standard contained 0.05 mg. per 13 cc. For convenience in reporting we have given all the readings in Table II as taken against a 0.05 mg. uric acid standard. It has been the

TABLE II.

Amount of substance X.	Reading of standard (0.05 mg. uric acid in 13 cc.).	Reading of test.	Calculated reading of standard when test is set at 15 mm.
<i>mg.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
0.50	15.0	4.9	45.1
0.40	15.0	5.3	42.5
0.30	15.0	6.5	34.6
0.25	15.0	7.4	30.4
0.20	15.0	8.6	26.2
0.10	15.0	12.2	18.4
0.08	15.0	14.5	15.5
0.05	15.0	19.3	11.6

usual custom with those determining uric acid to set the standard cylinder in the colorimeter, say at 15 mm., and move the cylinder in the test solution to match. We have in this case followed the customary procedure, but for purposes of graphical representation we have calculated the corresponding readings of the standard when the test cylinder is set constantly at 15 mm., as it is easier to see any divergence from proportionality when the ideal graph is a straight line instead of a logarithmic curve. From Table II Chart 1 (A) has been constructed. We see at once that from 0.5 to 0.05 mg. of substance X the colour produced is markedly disproportional to the amount of substance present. Herein arises the necessity for the readings of standard and test to coincide approximately, preferably in a relatively weak solution still strong enough to read easily. As may be seen from the graph the same

amount of colour is produced by 0.05 mg. of uric acid as by 0.075 mg. of substance X, so that 1 part of uric acid is equivalent to 1.50 parts of substance X. That is, the values obtained from blood calculated in terms of uric acid must be multiplied by 1.50 to express them as substance X *when the readings are taken under the conditions we have specified*. When, however, a colour is obtained by the French method of estimation which approximately matches that of a uric acid standard containing 0.1 mg., the factor necessary to convert to substance X is 2.4. This factor increases and becomes less reliable as the concentration of substance X increases.

We have estimated that about 80 per cent of the colour produced by the French reagents in certain animal bloods is due to

TABLE III.

Amount of substance X.	Reading of standard (0.01 mg. uric acid in 15 cc.).	Reading of test.	Calculated reading of standard when test is set at 15 mm.
<i>mg.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
0.20	15.0	6.1	36.9
0.15	15.0	7.6	29.6
0.10	15.0	10.5	21.4
0.075	15.0	15.2	14.8
0.05	15.0	20.9	10.8

substance X, and this same substance is probably responsible for about the same proportion of non-uric acid colour in human blood. So that if we take a French value of 10 mg. per 100 cc. of whole blood, the actual amount of substance X present will be about 12 mg. per 100 cc. A conservative estimate would thus place the concentration of substance X in human corpuscles at 25 mg. per 100 cc. Of course, as we have already mentioned, the concentration of substance in the blood of the same individual is subject to a wide range of variation. We have found values as often above as below 10 mg. per 100 cc. of human whole blood. Thus it can safely be said that at least 4 mg. of corpuscle "rest nitrogen" become accounted for, as substance X contains 17.5 per cent nitrogen.

Colour Values of the Pure Substance by the Direct Method.

The colour produced by different amounts of substance X was now compared with that from uric acid by employing the direct procedure of Benedict (1). The amount of substance X taken ranged from 0.20 to 0.05 mg. When quantities from 0.20 to 0.10 mg. of substance X were employed the colours produced were matched against a 0.02 mg. uric acid standard; when less than this, a 0.01 mg. uric acid standard was used.

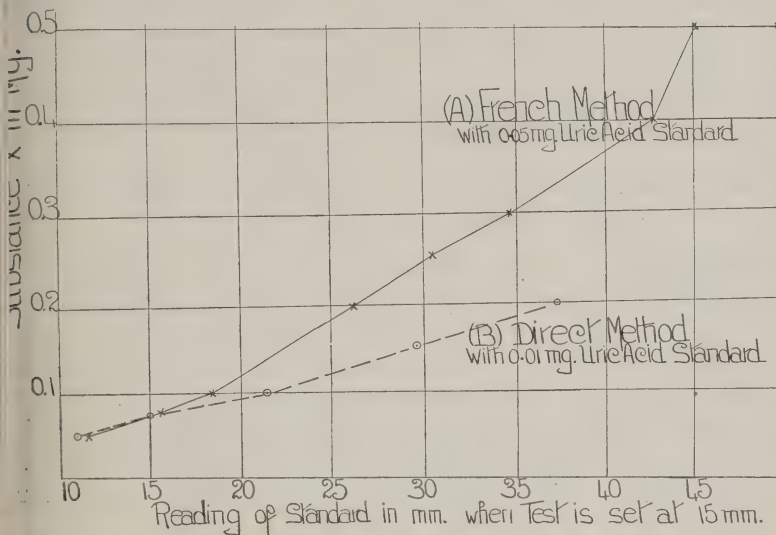


CHART 1.

The readings are all calculated in Table III as against a 0.01 mg. uric acid standard, and likewise for graphical purposes the readings of the standard are calculated when the test is constantly at 15 mm.

It will be seen from Chart 1 (B) that there is a much better proportionality even between the limits of 0.20 and 0.05 mg., than is given by the French method. It is further found from Table II that 0.075 mg. of substance X gives practically the same intensity of colour as 0.01 mg. of uric acid, so that 1 part of uric acid produces the same amount of colour as 7.5 parts of substance X. It was shown that by the French method 1 part of uric acid was equivalent to only 1.50 parts of substance X so that the

cyanide method increases the sensitivity of uric acid without appreciably affecting the sensitivity of substance X.

In view of the fact already noted that the French method does not measure a single substance in blood, we should not expect an exact relationship between the direct and the French values in the animal bloods in Table I. For if there were only one substance responsible for the reduction of phosphotungstic acid in uric acid-free bloods, and if the values were determined as we have indicated, we should find that the direct value multiplied by 7.5 would be equal to the French value multiplied by 1.50. This calculation applied to the few values given in Table I furnishes strong evidence that the colour given by the French method is largely due to the substance giving the colour in the direct method.

Effect of the Presence of Uric Acid on the Development of Colour from Substance X by the Direct Method.

That substance X is mainly responsible for the difference in the values obtained by the direct method and by the indirect method (according to Folin and Wu) on human bloods, is rendered highly probable, to say the least, by the following considerations.

Human bloods, it would appear, do not give such large divergences as are obtained by the two methods in animal bloods for the reason that uric acid inhibits the production of colour by substance X. This is well shown in the following experiments.

0.20, 0.10, and 0.05 mg. of substance X were added to three tubes containing 0.02 mg. of uric acid, the requisite water and reagents were added and the three tubes were heated along with a tube containing 0.02 mg. of uric acid. With the standard set at 15 mm. the tests read respectively at 8.0, 10.5, and 13.5 mm. In the first case, calculating from the 8.0 mm reading:

	mg.
Total observed as uric acid	=0.0375
Uric acid present	=0.0200
But from Table III, 0.2 mg. of substance X gives reading of 6.1 mm. against 0.01 mg. of uric acid standard, so that substance X should give colour expressed as uric acid	=0.0246
So total present as uric acid	=0.0446
Loss as uric acid is thus 0.0446 - 0.0375	=0.0071
So that substance X accounted for as uric acid is 0.0246 - 0.0071	=0.0175

Originally 0.20 mg. of substance X was added. Assuming this amount in 5 cc. of blood filtrate, the amount in the blood would be 40 mg. of substance X per 100 cc. But in the presence of 4.0 mg. of uric acid per 100 cc., only $0.0175 \times 200 = 3.5$ mg. would be accounted for by the cyanide method.

In the presence of 0.1 mg. of substance X the following values are obtained.

	<i>mg.</i>
Total observed as uric acid	=0.0286
Uric acid present	=0.0200
Substance X as uric acid	=0.0143
Total present as uric acid	=0.0343
Loss as uric acid	=0.0057
Substance X accounted for as uric acid	=0.0086

0.1 mg. of substance X in 5 cc. of blood filtrate is equivalent to 20 mg. of substance X per 100 cc. of blood, but in the presence of 4.0 mg. of uric acid per 100 cc. of blood, only $0.0086 \times 200 = 1.72$ mg. would be accounted for.

In the presence of 0.05 mg. substance X the following values are obtained.

	<i>mg.</i>
Total observed as uric acid	=0.0222
Uric acid present	=0.0200
Substance X present as uric acid	=0.0072
Total present as uric acid	=0.0272
Loss as uric acid	=0.0050
Substance X accounted for as uric acid	=0.0022

0.05 mg. of substance X in 5 cc. of blood filtrate is equivalent to 10 mg. of substance X per 100 cc. of blood, but in the presence of 4.0 mg. of uric acid per 100 cc. of blood only $0.0022 \times 200 = 0.44$ mg. as uric acid would be accounted for.

The presence of 10 mg. of substance X along with 4.0 mg. of uric acid best represents the distribution of these constituents in normal human bloods. The fact that the equivalent of 10 mg. of substance X per 100 cc. of blood raises the value given by the direct method by 0.44 mg. above the uric acid actually present, coincides very remarkably with the common finding that the direct method gives uric acid values for whole blood about 0.5 mg. higher than those given by the indirect method; and verifies the contention of Bulmer, Eagles, and Hunter that this increment by the Benedict method is not due to loss by manipulation in the Clin and Wu procedure.

It is convenient to refer here to a recent criticism by Benedict (3) of the paper by Bulmer, Eagles, and Hunter (4). Only one point in that criticism calls for attention, regarding the statement by Bulmer, Eagles, and Hunter that substance X is not

precipitated by silver lactate. As Part II of this paper shows substance X is precipitable by silver nitrate in acid solution. The statement by Bulmer, Eagles, and Hunter was admittedly inaccurate, or rather, incomplete, as it should have been followed by the further statement, "or not freed from its silver compound by the sodium chloride and hydrochloric acid mixture of Folin and Wu." The Folin and Wu procedure as a whole was the only point that concerned the findings of Bulmer, Eagles, and Hunter and the only one of consequence in the determination of uric acid. That procedure separates uric acid from substance X, as was shown by Bulmer, Eagles, and Hunter, and this fact is even more unmistakably shown by the use of phosphotungstic acid in presence of sodium carbonate instead of sodium cyanide.

PART II.

Preparation of Substance X from Blood.

From about 2.5 gallons of fresh pig's blood 5.5 liters of corpuscles were obtained by centrifugation. Two volumes of water and one volume of 0 normal sulfuric acid were added, and the temperature of the mixture was raised, with constant stirring, to about 75°C. when a drop on the end of the stirring stick was clear and almost colourless. The hot mixture was poured through cheese-cloth and the residue squeezed in a filter press. The residue was washed in the same manner with 5 liters of water. The total filtrate was run through a Büchner funnel to rid it from particles which had passed the cheese-cloth. About 3 cc. of glacial acetic acid were added to render just acid the clear reddish-coloured fluid amounting to 22 liters which was then cooled under the tap.

To remove the remaining protein, a saturated aqueous solution of uranic acetate was added until precipitation ceased and a drop of the clear supernatant gave a faint brown colour with potassium ferrocyanide. About 1700 cc. of the uranic acetate were required. The whole was then filtered through large gravity funnels allowed to drain overnight. The volume of the filtrate was 22.7 liters.

To remove more undesirable material a saturated aqueous solution of neutral lead acetate was then added to the filtrate to maximum precipitation and a neutral or faintly alkaline reaction to litmus. About 900 cc. of the lead acetate were required. The mixture was well shaken, the precipitate given about an hour to settle, and the almost clear supernatant siphoned into another vessel. Additional fluid was obtained by centrifugation of the lead precipitate which was discarded.

About 250 cc. of saturated alcoholic mercury chloride were found sufficient to precipitate completely the collected fluids. The mercury precipitate coagulated well and soon settled, leaving a water-clear supernatant

which was siphoned off. The precipitate was centrifuged and well washed with water. It was then suspended in about 400 cc. of water, the mercury removed as sulfide, and hydrogen sulfide removed from the filtrate by a current of air.

The volume of filtrate was then made to about 1500 cc. with water, and excess, about 250 cc., of 20 per cent aqueous solution of sugar of lead added, and then 2.5 normal sodium hydroxide to distinct alkaline reaction to litmus. The precipitate was filtered off and the volume of filtrate measured.

An aliquot portion, 10 cc., was taken in a centrifuge tube and 2.5 normal sodium hydroxide added in small portions until a bulky, well coagulated precipitate appeared on shaking. The amount of soda added was noted. The tube was centrifuged. 1 cc. of the clear supernatant was found to give no colour with phosphotungstic acid and sodium carbonate, but a small amount of the precipitate gave a deep blue colour with these reagents. The calculated amount of sodium hydroxide required for the whole filtrate was found to be 90 cc.; it was added, the mixture was well shaken, then centrifuged, and the precipitate washed with water.¹

The precipitate was then ground in a mortar with sulfuric acid until an excess was present as shown by testing the filtrate with barium chloride. Lead sulfate was filtered off and the filtrate made almost neutral to litmus with 2.5 normal sodium hydroxide.² A solution of mercury sulfate in sulfuric acid (Hopkins and Cole reagent) was then added to the filtrate, at this stage amounting to about 400 cc., until there was no further precipitation. The mercury precipitate was centrifuged, washed with water, suspended in about 100 cc. of water, and the mercury freed with hydrogen sulfide. The mercury sulfide was filtered off and the filtrate freed from hydrogen sulfide by a current of air.

The filtrate was made 0.5 normal acid by the addition of the requisite quantity of sulfuric acid, and a 20 per cent solution of phosphotungstic acid in 0.5 normal sulfuric acid was added until precipitation ceased. The pinkish precipitate was allowed to stand overnight in the ice chest. It was then centrifuged and thoroughly washed with cold 0.5 normal sulfuric acid to which a few drops of phosphotungstic acid had been added. It was then transferred to a mortar, suspended in about 30 cc. of water, and ground with an excess of barium hydroxide powder. The alkaline mixture was filtered in a Büchner funnel and the filtrate carefully freed from excess of barium and sulfuric acid. The final filtrate was neutral to litmus and had

¹ This lead precipitation requires some care but is the most important step in the isolation of substance X. The adjustment of the requisite amount of sodium hydroxide is sometimes a little difficult as the precipitate sought readily dissolves in excess of alkali, but we have found the above technique so useful that baryta and ammonia, which may be less specific, have not been tried.

² If at this stage there is any sign of precipitation on approaching neutrality, continue addition of soda to maximum precipitation, filter off the precipitate, and bring back the reaction to faint acidity with sulfuric acid.

only a trace of colour, which was removed by the addition of a little charcoal and a few minutes' immersion in a boiling water bath. The water-clear filtrate from the charcoal was then evaporated *in vacuo* until signs of solidification appeared, at which point the volume was reduced to 2 to 3 cc. While still hot, about 20 cc. of absolute alcohol were added. The substance separated quickly but not suddenly in a microcrystalline condition. The containing tube was allowed to stand overnight in a refrigerator, then the contents were filtered through a small Gooch filter by suction. The substance was washed with absolute alcohol and dried in an evacuated desiccator containing calcium chloride. The yield from 5.5 liters of corpuscles was 0.7 gm. of perfectly white substance, the microscopic appearance of which is shown in Figs. 1 and 2.

A silver fractionation was employed in another extraction. Thus, to the phosphotungstic acid filtrate freed from barium and sulfuric acid, 20 per cent aqueous silver nitrate was added until there was no further precipitation. A dense and exceedingly gelatinous precipitate was obtained which readily goes into colloidal solution on repeated washing with water. The once washed precipitate was suspended in water and hydrogen sulfide passed. Colloidal silver sulfide was thus formed which was coagulated only with difficulty, and the phosphotungstic acid precipitation had again to be applied before final isolation of the substance.

Apart from the difficulties of the silver technique no purpose appears to be served by this additional step, as one preparation obtained by it had the same m.p. as three others prepared without its help.

In order to get as good a yield as 0.7 gm. from 5.5 liters of corpuscles, which represents about 13 mg. per 100 cc. of corpuscles and which is probably not more than a fourth to a third of that actually in pig's corpuscles, certain general precautions have to be taken. Speed in working appears to be necessary, especially when the substance is in an alkaline medium. We have found that 3 to 4 days are necessary to obtain the substance in the pure state and that it is safest to leave it overnight at the following stages: the uranium acetate filtrate, the mercury precipitate in presence of hydrogen sulfide, and the phosphotungstate in acid solution. After this stage we have found little spontaneous decomposition of the substance, but before it, for reasons yet unknown to us, very considerable losses may occur.

Properties of Substance X.

When heated for 2 hours in a steam bath at 100° and placed in a vacuum desiccator overnight there is no loss of weight. The substance has thus neither water nor alcohol of crystallisation.

When the substance is heated in a capillary tube slight browning occurs about 250°. This colour intensifies until a very sharp m.p. is reached at 269 to 270°. Specimens from four different preparations had the same m.p.

The substance was found to be free from phosphorus and sulfur.

Nitrogen was estimated by Kjeldahl with the aid of potassium sulfate and a small piece of copper.

0.1051 gm. of substance yielded 17.58 per cent nitrogen.

The average of two micro Kjeldahl determinations gave 17.50 per cent nitrogen.

Carbon, Hydrogen, and Oxygen.—For the determination of carbon and hydrogen we were fortunate in having the skilled services of Professor L. J. Rogers of the Department of Analytical Chemistry. We desire to thank Professor Rogers for the following data.

Substance.	CO ₂	H ₂ O
<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
0.2169	0.3623	0.1300
0.2484	0.4117	0.1540
C	H	N
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
45.55	6.66	
45.21	6.84	
Average.....45.38	6.75	17.54
	C ₆ H ₁₁ N ₂ O ₃	
	Calculated.	Found.
C	45.28	45.38
H	6.92	6.75
N	17.61	17.54
O	30.19	30.33

The *molecular weight* was determined in aqueous solution by depression of the freezing point. 25 mg. substance in 4 gm. of water gave a depression of 0.060° as an average of two readings, which gives a molecular weight of $\frac{1860 \times 0.025}{0.060 \times 4} = 194$. The addition of a further 25 mg. of substance showed an average depression from two readings of 0.118°, which gives a molecular weight of $\frac{1860 \times 0.050}{0.118 \times 4} = 197$. On the assumption of an addition of two molecules of water in aqueous solution the calculated molecular weight for C₆H₁₁N₂O₃ · 2H₂O is 195. The empirical formula of substance X is thus C₆H₁₁N₂O₃.

Specific Rotation.—0.1500 gm. of substance was dissolved in 14.85 gm. of water from which was filled a 2 dm. tube. The average of twelve readings showed a levorotation of 2.299° . At twice the dilution the average of six readings showed a levorotation of 1.150° . The specific rotation of substance X is thus:

$$[\alpha]_D^{27.5^\circ} = \frac{-2.30^\circ \times 100}{2 \times 1} = -115.0^\circ$$

Amino nitrogen was tested for by the micro method of Van Slyke. 15 mg. of substance were put into the Van Slyke micro apparatus, mixed with the reagents in the bulb, allowed to stand for 15 minutes, and then shaken for 2 minutes. The result found was 1.32 per cent expressed as amino nitrogen.

On repetition with the same amount of substance but allowing to stand for 30 minutes 1.53 per cent amino nitrogen was obtained. It is thus assumed that the substance contains no amino nitrogen.

The substance has the further properties:

It is neutral to litmus, very soluble in water, and slightly soluble in hot alcohol.

It gives a blue colour with phosphotungstic acid in presence of sodium carbonate and also with arsenophosphotungstic acid in presence of sodium cyanide. (See above.)

On the addition of bromine water there is a temporary precipitate and rapid decolorisation of the solution. When fully oxidised with bromine the blue colour with the uric acid reagents ceases to be given.

In presence of sulfuric acid and in presence of potassium hydroxide, potassium permanganate is rapidly decolorised.

In neutral or acid solution iodine in potassium iodide is rapidly decolorised. In solution as dilute as 0.1 per cent a heavy precipitate is formed on the addition of the iodine solution.

The behaviour with bromine, iodine, and permanganate indicates unsaturation in the molecule.

A heavy dense brown precipitate is given by gold chloride in presence of hydrochloric acid.

Picric acid gives a precipitate from relatively strong solutions.

A yellow colour is obtained from extremely dilute solutions with the diazo reagents of Koessler and Hanke (17). The intensity of colour is not proportional to the amount of substance present.

Weyl's test is positive. To a solution add a few drops of sodium nitroprusside and make alkaline with sodium hydroxide. There is no primary colour. Let stand a few minutes and then acidify with acetic acid when a bluish green colour develops rapidly in the cold.

In dilute solutions the substance gives a strong colour with picric acid and sodium hydroxide in the proportions used for the determination of creatinine. This colour shows up distinctly only after standing for several hours. Substance X is thus probably one of the factors which make the present method for the determination of creatinine in corpuscles difficult. (See Hunter and Campbell (15).)

The iodoform test with iodine and potassium hydroxide is positive in the cold.

The substance is extremely resistant to the action of acids. Thus, heating 75 mg. of substance with 20 per cent sulfuric acid for 4 hours at 140° did not appreciably diminish the blue colour produced by phosphotungstic acid and sodium carbonate. Only after heating for a further $4\frac{1}{2}$ hours with 20 per cent sulfuric acid at a temperature of 175° did the solution cease to give any blue colour with the uric acid reagents.

The following tests were negative. Protein tests; reducing tests with Benedict's qualitative reagent for sugars, and the chloroglucinol and orcinol tests for pentose; murexide test and Millon's test. No colours were given with ferric chloride, potassium persulfate, or nitroprusside and ammonia.

The Probable Chemical Nature of Substance X.

We have as yet insufficient data to justify any graphical representation of the molecular structure of substance X but have grounds for believing that it is a simple pyrimidine nucleoside. Its behaviour with lead and soda and its resistance to hydrolysis by acids favour this view. No known purine or pyrimidine nucleoside fits the empirical formula or gives the same m.p. as substance X, although a mononucleoside of thymine synthesised by Johnson and Chernoff (16) is very suggestive from this point of view. According to these workers their substance has the empirical formula $C_6H_8N_2O_3$, a m.p. of $224-225^{\circ}$, and does not undergo hydrolysis to thymine and formaldehyde when heated at 140° with sulfuric acid.

Little emphasis can at present be attached to the iodoform test which probably indicates an alkyl group in the molecule.

If substance X contains a pyrimidine nucleus common to the nucleic acids it would seem that cytosine is definitely excluded on account of its three atoms of nitrogen. The fact that substance X is precipitable by phosphotungstic acid favours uracil, but other constituents of the thymine molecule might lead to an insoluble phosphotungstate.

SUMMARY AND CONCLUSIONS.

A new substance of the empirical formula $C_6H_{11}N_2O_3$, believed to be a simple pyrimidine nucleoside has been isolated from pig's blood, and evidence has been given of its presence in other animal bloods as well as in human blood. It is commonly present in human whole blood to the extent of 10 to 12 mg. per 100 cc. and as it is confined entirely to the corpuscles it is there in approximately twice these concentrations. The amount in blood varies over a wide range even in the same individual.

The substance gives a blue colour with the phosphotungstic and arsenophosphotungstic acid uric acid reagents in presence of either sodium carbonate or sodium cyanide. The presence of the substance in human blood is made more conspicuous by the use of sodium carbonate, as with this alkali the reagent is less sensitive towards uric acid.

The behaviour of the new substance has been quantitatively studied with the old and new uric acid reagents and the conclusion has been reached that the discrepancies between the direct method of Benedict and the precipitation method of Folin and Wu for the estimation of uric acid in human blood are due to the fact that this new substance adds to the values obtained for uric acid by the direct method. The substance which we have now isolated is that mainly responsible for the direct "uric acid" values recently obtained in animal bloods by Bulmer, Eagles, and Hunter, and is separable from uric acid in blood filtrates by the precipitation method of Folin and Wu.

Our thanks are due to Mr. R. M. Rowatt of the Canadian Packing Company, Toronto, for supplying us with material, to Miss V. Gillett for the photographs, and again to Professor

Rogers for analytical data. We also wish to express our thanks to the Medical Research Committee of this University for access to their animals, and our appreciation of Professor V. J. Harding's interest in the work throughout.

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EXPLANATION OF PLATE 2.

FIG. 1. Photomicrograph of substance X. Low power.

FIG. 2. Photomicrograph of substance X. High power.



FIG. 2.

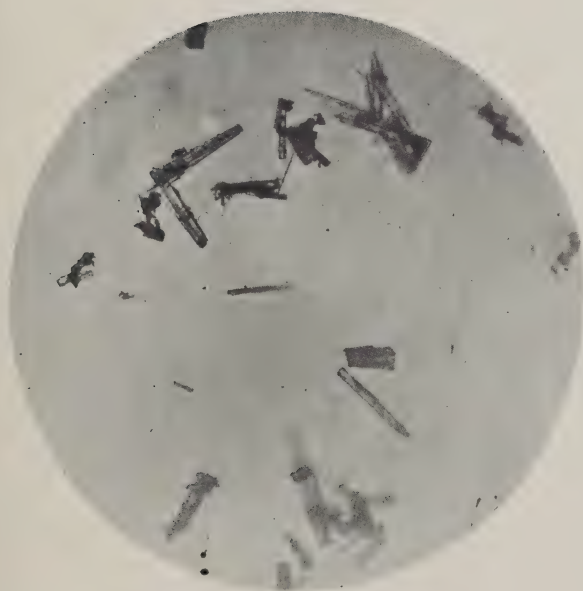


FIG. 1.

(Hunter and Eagles: A new substance from blood.)

THE INFLUENCE OF SUNLIGHT ON BONE DEVELOPMENT IN SWINE.

By L. A. MAYNARD, S. A. GOLDBERG, AND R. C. MILLER.

From the Department of Animal Husbandry and the Department of Comparative Pathology and Bacteriology, Cornell University, Ithaca.)

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A report has recently been made by the writers (1) on the dietary relationships and the pathology of a condition in swine popularly referred to as stiffness, paralysis, rickets, and by other terms. In this previous study the principal and constantly occurring lesions in the stiff pigs were found in the bones and these lesions were accompanied by a deficiency of calcium and phosphorus as shown by chemical analysis of the femurs. The stiffness was found to occur with a ration low either in calcium or in the factor aiding its assimilation. The correction of the ration with respect to these deficiencies was found effective for preventing or curing the trouble.

In this previous study the animals were housed in pens on the north side of the colony house and were never exposed to any direct sunlight. In view of the findings that the stiffness was apparently a result of faulty mineral nutrition the question naturally arose as to how the results would be modified where the pigs were exposed to sunlight. The results reported in the present paper deal with a study of this question.

EXPERIMENTAL.

The study consisted of two trials—one conducted in the summer of 1924, and one carried out the following winter.

The rations used are shown in Table I.

These rations contain digestible crude protein and total digestible nutrients in the proper relation for young pigs according to accepted feeding standards—though the protein, furnished entirely by plant sources, is not of sufficiently high quality for a maximum

rate of growth. In other respects the rations are believed to be adequate for the growth and normal development of swine with the exception of certain deficiencies with respect to mineral nutrition. The basal ration is very deficient in calcium. This deficiency is corrected in the other ration by the addition of the sources of calcium listed, thus making this ration approximately nine times higher in calcium than the basal ration and somewhat higher in phosphorus. Both rations are presumably low in the factor aiding calcium assimilation.

These same rations were used by the writers (1) in their previous study in which the pigs were so housed as to have no access to

TABLE I.
Composition of Rations.

Ration.	Ingredients.	Calcium.	Phosphorus.	Nutritive ratio.
		<i>per cent</i>	<i>per cent</i>	
Basal.	200 lbs. yellow corn-meal.	0.096	0.55	1:4.8
	100 " wheat middlings.			
	75 " oil meal.			
" plus minerals.	200 " yellow corn-meal.	0.795	0.71	1:4.8
	100 " wheat middlings.			
	75 " linseed oil meal.			
	4 " steamed bone meal.			
	4 " ground limestone.			

direct sunlight. The basal ration nearly always resulted in the development of the characteristic stiffness within 4 months, while on the ration with the added minerals no cases of stiffness resulted during the same period. The bones of the pigs on the latter ration showed a markedly higher content of calcium and of phosphorus and a more nearly normal histological picture than did the bones from the pigs on the basal ration.

The primary object of the study here reported was to ascertain whether the exposure of pigs fed the basal ration to sunlight would prevent the development of the stiffness, and result in a better assimilation of the limited amount of calcium present than would result in a similar group of pigs on the basal ration receiving no direct sunlight.

The pigs used were pure bred Duroc-Jersey barrows farrowed in the University herd. They weighed from 20 to 30 pounds when placed on experiment. Only thrifty pigs were used and they were chosen with the object of having as many litter mates as possible distributed among the groups to be compared. By this method of distribution, opportunity was afforded for comparing the bones of litter mates, thus eliminating differences due to previous nutritional history and breeding. Since the litter mates had been fed like from birth to the time they were placed on experiment and were approximately equal in weight at this time, it was considered that the variable factor of stored nutrient reserves was reduced to a minimum. At the start of the experiment all of the pigs were given two treatments of oil of chenopodium for intestinal parasites.

Trial 1.

On June 10, 1924, four pigs were placed on each of the rations listed in Table I in pens on the north side of the colony house. A third group of four was placed on the basal ration in a pen on the south side of the house, opening on a cement runway, 7 by 12 feet. Thus this group was permitted to go outside at will and actually spent a considerable portion of each sunny day outdoors. On the other hand the other groups were never outside and were never exposed to any direct sunlight. The group which was allowed outdoors had somewhat larger quarters by reason of the runway, but it is not believed that the other two groups were hampered in activity or in other respects by the size of their quarters. The group in the pen with the runway was somewhat more active but it is believed that this was due to the stimulus of being outside rather than to their larger quarters.

The two groups on the basal ration, one with access to sunlight and the other not, furnished an opportunity for ascertaining whether the sunlight would increase the assimilation of calcium on this low calcium ration, as judged by comparative chemical and histological studies of the bones. The group on the basal ration plus the added sources of calcium was included in the trial with the expectation, based on previous results, that nearly normal bones could thus be available as a further basis for comparison in studying the effect of the sunlight.

The pigs were fed all they would eat three times a day. They were weighed periodically and were constantly watched for the development of the characteristic stiffness.

One of the pigs of the no-sunlight group on the basal ration developed the stiffness toward the end of the 3rd month and the other pigs in this group became stiff during the 4th month. On the other hand, none of the pigs exposed to sunlight showed any signs of the trouble at the end of the 4th month, nor did any of the animals receiving the ration with the added minerals.

With the exception of the first pig to become stiff, which was killed for examination at the end of the 3rd month, the animals in the no-sunlight group were held on the experiment until the close of the 4th month, by which time the stiffness had become greatly aggravated. At this time both these stiff pigs and their litter mates in the other two groups were killed for comparative study. Each animal was given a general pathological examination and then one femur was taken for histological study, while the other femur was reserved for chemical analysis.

Bone Analysis.—For the chemical analysis the femur was freed from adhering flesh, partially dried at 50° to 60°C. and then dried to constant weight over sulfuric acid. The bone was next pulverized in a bone cutter, sampled, and the sample ignited at dull redness in an electric muffle furnace to determine the ash. The results are shown in Table II.

Pigs 651, 654, and 652 were litter mates killed on the same day. In the last column of the table it is shown that the femur of the pig exposed to sunlight had a much higher ash content than that of its litter mate which became stiff on the basal ration without sunlight, indicating that the sunlight caused an increased assimilation of the very limited amount of calcium supplied in the ration. The bone of the litter mate on the ration containing the added sources of calcium is seen to be the highest in ash of the three.

A similar relationship is seen to exist for the next three litter mates—Nos. 631, 634, and 635. As noted from the figures for age, the stiff pig, No. 631, was killed 46 days earlier than the other two. This was the pig that became stiff much sooner than any of the others and which was autopsied at the end of the 3rd month. Since one object of the study was to ascertain the influence of sunlight in preventing the development of the stiffness, it seemed

desirable to give the litter mates of No. 631 further opportunity to develop the trouble. In so doing the comparison of the results of the bone analysis in the case of these three litter mates was rendered open to the criticism that ash content increases with age. It is not believed however that the fact that Pig 634 was 194 days old when killed while Pig 631 was only 148 days old could possibly explain the increase of approximately 60 per cent in the ash content of the bone of the former.

TABLE II.
Ash Content of Femurs.

Pig No.	Age.	Live. weight.	Ration.	Weight of fresh bone.	Composition of fresh bone.	
					Dry matter.	Ash.
	<i>days</i>	<i>kg.</i>		<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
651	196	41.3	Basal.	127.4	44.35	13.61
654	196	50.0	" + sunlight.	147.2	56.45	19.22
652	196	38.1	" + minerals.	120.5	57.59	22.25
631	148	26.8	"	96.5	44.15	11.25
634	194	50.3	" + sunlight.	164.0	61.10	18.16
635	194	45.4	" + minerals.	140.0	58.00	22.27
619	220	50.0	"	146.2	51.37	13.74
603	225	55.3	" + sunlight.	170.6	60.14	16.93
665	204	56.5	" + minerals.	187.0	62.62	22.65
609	189	45.8	"	148.0	50.74	17.18

Pigs 619, 603, and 665 were not litter mates but are grouped together in the table because they were approximately of the same size and age at the start of the experiment and were nearly the same age when killed. Here again with the basal ration the beneficial effect of the sunlight in increasing the ash content is seen, and again the highest ash content resulted where minerals were added.

Pig 609 was the fourth pig that became stiff on the basal ration fed indoors. The remaining pig on each of the other rations which was scheduled to be killed for comparison with No. 609 was particularly desired for teaching purposes at the time and thus was not slaughtered. This was seen to be unfortunate when the

ash content of the bone of No. 609 was determined. This pig was very stiff when killed, but the table shows the ash content of its femur to be markedly higher than the figure for any of the other stiff pigs and to be comparable with the figures for the pigs in the sunlight group. Even though the figure for No. 609 is thus comparatively high, it is entirely possible that its litter mate in the no-sunlight group developed a bone of still higher ash content. In the absence of the opportunity to check this possibility because of the previously mentioned failure to kill the litter mate in question the figure for No. 609 must stand as an apparent exception to the otherwise consistent results in Table II.

It is seen in Table II that the pigs exposed to the sunlight were uniformly heavier when killed than the pigs on the same ration kept inside. This observation, which may seem to indicate that the sunlight had a beneficial effect on growth as measured by body weight, is of limited significance because the pigs fed the basal ration inside usually remained stationary in weight or declined during the period intervening between the beginning of the stiffness and the time of slaughter. It is true that the periodic weighings indicated that, as a whole, the sunlight group grew faster, but no weight records are reported because it was not the intention to make any comparison of rate of growth, nor could such a comparison be significant with the small number of animals involved.

The figures for "weight of fresh bone" in Table II show that the bones were uniformly heavier for the pigs in the pen with the outdoor runway as compared with those inside on the same ration. Similarly the bones of the pigs exposed to the sunlight were higher in dry matter. These figures for dry matter have been included in the table primarily to indicate, as can be proven by calculation, that the conclusions drawn as to ash content would not be affected if the figures for ash were compared on a moisture-free basis.

Pathological Examinations.—On routine pathological examination, the stiff pigs in the no-sunlight group showed hemorrhage in groups of lymph nodes, and the kidneys or the mucosæ of the urinary bladders contained petechiæ. The same changes were found in the pigs in the other groups but to a markedly lesser degree.

Pneumonic lesions were found in four of the animals: Nos. 654,

652, 634, and 609. Since three out of four of the stiff pigs, namely Nos. 651, 631, 619, were free from pneumonia it is clear that this disease was not a complicating factor affecting the definiteness of the results obtained.

On examination of the femurs it was found that the bones of the pigs in the no-sunlight group were markedly less dense, as indicated by the ease of sawing, than the bones of the pigs in the other two groups. The changes found for the femurs of the stiff pigs, both in the gross and under the microscope, were similar to those previously reported by the writers (1). The bone marrow was reddened throughout, or near the epiphyseal cartilage. The latter was irregular and much thicker than normal. The cortical bone was soft and porous. Under the microscope the constant lesions found were: imperfect calcification, granulation tissue, degenerated and proliferating areas of articular cartilage, osteoclasts along the trabeculæ, irregularity in the zone of provisional calcification, and hemorrhage under both the articular and epiphyseal cartilages.

The femurs of the pigs on the basal ration receiving the sunlight were more nearly normal in all cases than were the femurs of their litter mates in the no-sunlight group, just described. The reddening in the shaft was much less and the epiphyseal cartilage was thinner and more regular in all cases. The articular cartilage was normal. The cortical bone was denser and of a finer texture. Under the microscope there were much less hemorrhage and granulation tissue than were found in the bones of the litter mates on the basal ration. Similarly there were fewer areas of imperfect calcification and the zone of provisional calcification was more nearly normal.

Trial 2.

The second trial was begun November 21, 1924, and extended over a 4 months period. The study was repeated, not only to secure further data, but also to ascertain whether the beneficial effect of sunlight which was indicated in the trial carried out in summer would hold for winter conditions. In practice, stiffness was much more prevalent in winter than at other seasons. A partial explanation of this lies in the fact that the ration is apt to be more restricted in the winter time. However, even with similar rations

and with the animals always housed inside, the writers (1) in their previous study found that a larger percentage of stiffness occurred in winter.

Of more direct significance with relation to the present study is the fact that the winters are so severe in this latitude that on many days the weather is not suitable for the pigs to remain long outdoors. Further, there are fewer sunny days. Another consideration that has an important bearing is the fact that, as shown by Dorno (2), the ultra-violet solar radiation is much lower in winter than in summer.

The second trial was limited to a comparison of a group on the basal ration fed inside and a group on the same ration in the pen connected with the runway outdoors. The pigs in this latter group were fed out in the runway to make sure that they actually went outside at least three times a day. During the coldest weather the trap-door connecting the pen with the runway was kept closed except at feeding time, lest the excessive cold and exposure result in pneumonia and the significance of the data be limited thereby. With the exception of approximately 15 days the trap-door was always open and the pigs spent a varying amount of time outdoors, depending on the weather. During the latter part of February and early March a period of unseasonably warm weather resulted in the pigs spending a relatively large proportion of each day out in the runway towards the end of the trial. However, for the trial as a whole the average number of hours per day that the pigs were exposed to sunlight was certainly very much less than was the case in the previous summer. According to the records of the Ithaca office of the United States Department of Agriculture Weather Bureau, the average number of hours of sunlight per day for the period covered by the summer trial was 8.96 and for the winter trial, 4.87.

In Trial 2 every pig in one group had a litter mate in the other. Two of the pigs in the no-sunlight group became stiff toward the end of the 3rd month of the experimental period and were very stiff when they were killed early in the 4th month. The other two pigs in this group were slightly stiff by the middle of the 4th month. At the end of the month both were killed. One had become very stiff by this time, but in the case of the other one, No. 27, the stiffness had not become any more severe than when it was first noted.

The pigs in the sunlight group were killed at the same time as their litter mates previously mentioned. One of them, killed at the end of the 4th month, showed some indications of the characteristic stiffness, but the other three appeared entirely normal when slaughtered.

Bone Analysis.—Following the procedure of the previous trial the pigs were given a routine pathological examination as they were killed and then the femurs were taken for chemical and histological examination. The results of the chemical analyses are shown in Table III.

TABLE III.
Ash Content of Femurs.

Pig No.	Age.	Live weight.	Ration.	Weight of fresh bone.	Composition of fresh bone.	
					Dry matter.	Ash.
	<i>days</i>	<i>kg.</i>		<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
44	164	35.8	Basal.	124.9	49.89	11.55
46	164	36.7	" + sunlight.	121.5	57.20	17.12
43	190	42.2	"	170.5	53.61	13.12
42	184	50.0	" + sunlight.	182.6	62.65	19.05
31	164	36.7	"	120.7	52.11	16.25
37	164	42.2	" + sunlight.	146.2	63.71	18.82
27	170	38.1	"	125.0	51.92	18.58
26	164	43.5	" + sunlight.	152.2	55.52	18.62

It is noted in Table III that in the case of each of the first three pairs the ash content of the femur of the pig exposed to sunlight is markedly higher than that of its litter mate fed indoors. Thus the results are in agreement with those secured in Trial 1. In the case of the fourth pair, however, the ash content of the bone of Pig 26 receiving sunlight is no higher than that of its litter mate, Pig 27. While all of the other animals in the no-sunlight group were markedly stiff when killed Pig 27 was only slightly affected. From this fact it might be expected that the chemical analysis would show the bone of this pig to have a higher ash content than the bones of the other pigs on the basal ration, as was

actually the case. However, as is described further on in this paper, the pathological examination showed the changes to be more marked in the bone of Pig 27 than in that of its litter mate, the beneficial effect of the sunlight being evident with this pair as with the other pairs studied in this trial. Thus the results of the chemical analysis for Pigs 26 and 27 constitute an exception to the other results secured in Trial 2.

It is noted in Table III that, as was the case in the previous trial, the pigs receiving the sunlight were uniformly the heavier when killed and had heavier femurs in three out of four cases, and that these femurs were uniformly higher in dry matter.

Pathological Examinations.—On routine pathological examination the occurrence of hemorrhagic lymph nodes, and of petechia in the kidneys or mucosæ of the urinary bladders was noted as in the previous trial, the changes occurring to a much greater degree in the no-sunlight group. Pneumonia was found in only one case, Pig 31.

As in the previous trial the femurs of the pigs in the no-sunlight group showed the following characteristic changes in the gross bone marrow reddened throughout; spongy, porous cortical bone; an irregular and thickened epiphyseal cartilage, and hemorrhages under both the articular and epiphyseal cartilages. Under the microscope the zone of provisional calcification was irregular with a marked proliferation of cartilage cells. Granulation tissue was present instead of bone and there were a large number of osteoclasts along the trabeculæ. In the cortical bone under the periosteum there was granulation tissue instead of bone, and areas of hemorrhage in the marrow spaces of the medullary bone were noted.

As in the previous trial, the femurs of the litter mates in the sunlight group showed a pathological picture much less advanced. The cortical bone was denser, the epiphyseal cartilage was less irregular, and there was less hemorrhage. The changes in the zone of provisional calcification were less extensive. The changes involving the formation of granulation tissue and of osteoclasts along the trabeculæ did not extend as far into the diaphysis. In the case of Pigs 46 and 42 no granulation tissue was found while in Pigs 44 and 43 the formation had advanced to a considerable extent. Although in No. 26 the zone of provisional calcification

was irregular and there was hemorrhage under the articular cartilage as well as under the epiphyseal cartilage in the diaphysis, in No. 27 these changes were far more advanced and extended for a greater distance into the diaphysis. In the bone of Pig 31 more granulation tissue was present than was the case with Pig 37, bone calcification had not advanced as far, and the epiphyseal and articular cartilages were thicker.

Sections taken at the lumbar enlargement of the spinal cord of Pigs 46 and 31 were examined by Nissl's method and appeared to be normal except for a slight amount of hyperemia in individual vessels and a slight amount of coagulum in the central canal. One case of congestion of the pia-arachnoid of the spinal cord was previously reported by the writers (1) in their earlier publication. Otherwise, the former observations of the writers were confirmed.

DISCUSSION OF RESULTS.

The results of Trials 1 and 2 are in accord in showing that sunlight has a markedly favorable influence on the mineral nutrition of growing pigs fed a ration low in calcium and presumably low also in the factor aiding calcium assimilation. All of the pigs fed this ration without access to direct sunlight, a total of eight animals, developed the characteristic stiffness within 4 months, while seven out of the eight pigs receiving the same ration with sunlight showed no signs of this trouble over the same period. The remaining animal exposed to sunlight, a member of the winter group, was apparently slightly stiff at the end of the 4 months period.

The results in Tables II and III are in agreement in showing that the sunlight has in general a marked effect in increasing the ash content of the bones. This influence of the sunlight was clearly shown in six out of seven of the comparisons made.

It is believed that the comparisons between the litter mates constitute the best method of presenting and of judging the significance of the results secured. However, in view of the fact that in one comparison the sunlight was apparently without influence on the ash content, it is to the interest of accurate interpretation of results to ascertain how these figures, which are out of line with the rest, affect the significance of the mean values which may be computed for each group. Calculation shows that the

average ash content for the femurs of the eight pigs in the no-sunlight group is 14.41 per cent ± 0.701 , computing the probable error by Peter's formula. The average in the case of the seven pigs examined from the sunlight group is 18.28 per cent ± 0.257 . The difference between the averages thus becomes 3.87 per cent ± 0.747 , clearly a significant figure.

The results of the histological studies were in agreement with the findings of the chemical analyses in showing that a more nearly normal bone was produced where the animals were exposed to sunlight.

The bones of the animals in the sunlight group were by no means normal either in ash content or histologically, nor were they as good in these respects, according to Trial 1, as the bones from pigs receiving the basal ration plus the added sources of calcium, but not having access to direct sunlight. However, the bones of this last group were still below normal, both in ash content and histologically, as compared with the bones of pigs fed a ration rich in minerals on pasture, studies of which were previously made by the writers. Thus it would be reasonable to expect that a still better state of calcium nutrition would have resulted if a group had been fed the ration with the extra sources of calcium and exposed to sunlight also. In fact it has been recently shown both by Steenbock, Hart, and Jones (3) and by Zilva, Golding, and Drummond (4) that with a ration amply supplied with calcium and phosphorus but low in the antirachitic factor a better bone development will result where the animals are housed with access to sunlight.

A comparison of the results of the two trials shows no certain evidence that the sunlight produced any greater effect in summer than it did in winter. It is true that in the winter trial one of the comparisons did not show any advantage for the sunlight. Further, one of the pigs in this trial apparently became slightly stiff despite the sunlight. However, a comparison with a much larger number of animals than was used in the present study is needed to ascertain definitely whether certainly better results would be obtained in the summer. Clearly the summer group received much more sunlight and a sunlight of higher efficiency but the winter sunlight may have been sufficient in amount and efficiency to produce the maximum possible effect on assimilation of calcium, with the small amount of the latter supplied.

From a practical standpoint the results indicate that the common observation, that both growing pigs and brood sows keep in better condition and are less likely to develop stiffness on pasture than when they are housed inside, may be explained on the basis of sunlight as well as of feed. It is also indicated that it is good practice to let the pigs spend a part of each day outdoors, in winter as well as summer, whenever the weather is not too severe, and that healthier and better nourished animals may result thereby.

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SOME NITROGENOUS CONSTITUENTS OF THE JUICE OF THE ALFALFA PLANT.

VI. ASPARAGINE AND AMINO ACIDS IN ALFALFA.*

BY HUBERT BRADFORD VICKERY.

(From the Laboratory of the Connecticut Agricultural Experiment Station,
New Haven.)

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The presence of asparagine and tyrosine in the juice of the alfalfa plant was recently reported from this laboratory (1). The methods of protein analysis have since been applied to the examination of a larger quantity of material and, in addition to asparagine and tyrosine, alanine, valine, leucine, phenylalanine, and serine have been found in the fraction which contains the nonoamino acids.

Amides and amino acids have long been recognized as constituents of plant juices and nearly all of the protein "Bausteine" have been found in plants, mainly through the efforts of Schulze and his collaborators. Asparagine is one of the longest known nitrogenous constituents of plants and is widely distributed. It was first recognized by Delaville in 1802 in asparagus (2). Von Jorup-Besanez found leucine in vetch seedlings in 1874 and tyrosine in seedlings of *Vicia sativa* in 1877 (3). Schulze and Barbieri discovered phenylalanine in seedlings of *Lupinus luteus* in 1879, before it was found among the products of hydrolysis of proteins (4) and somewhat later found valine in the same material (5). So far as we can learn alanine and serine have not previously been reported as constituents of plant juices.

The material employed in the present analysis was the precip-

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itate obtained with Neuberg's reagent (6) when working up alfalfa filtrate for the betaine fraction, a report on which was recently published (7). The precipitate was decomposed with hydrogen sulfide and the solution treated with phosphotungstic acid in the usual way, thereby removing purines, basic amino acids, and, probably, some of the peptides. The filtrate contained the asparagine and amino acids. These operations were carried out as rapidly as possible so as to minimize hydrolysis of asparagine by the reagents. The distribution of nitrogen in the alfalfa filtrate and in the amino acid fraction obtained from it is given in Table I.

Only a little over one-half of the amide nitrogen of the alfalfa filtrate was found in the amino acid fraction. The nature of the substances yielding "amide nitrogen" by the customary method

TABLE I.

Analysis of Alfalfa Filtrate and of the Monoamino Acid Fraction Obtained from 23.96 Kilos of Fresh Alfalfa.

	Alfalfa filtrate.	Amino acid fraction.	Proportion in amino acid fraction.
	gm	gm.	per cent
Total nitrogen.....	55.13	12.46	22.6
Ammonia nitrogen.....	1.538	0.244	15.9
Amide nitrogen.....	5.775	3.026	52.4
Amino ".....	14.38	7.10	49.4
Solids (ash-free).....	870.4	114.6	13.2

of analysis in the precipitate obtained with barium hydroxide and alcohol removed as a preliminary step in the fractionation (see experimental part), and in the phosphotungstic acid precipitate, remains to be determined.

The amino acid solution was concentrated *in vacuo* and as much asparagine removed by crystallization as possible. In addition a small amount of tyrosine was obtained. The mother liquor was then boiled with 20 per cent hydrochloric acid to hydrolyze the remaining asparagine as well as the small amount of peptides which previous experience had shown to be present in this fraction (1). After removal of ammonia, the dibasic amino acids were precipitated as barium salts by means of alcohol, the monoamino acids in the filtrate esterified, and the esters distilled. The substances, in yields given in Table II, were isolated.

These substances account for 29.5 per cent of the solids and, together with the 1.66 gm. of ammonia nitrogen set free by the hydrolysis, for 54.8 per cent of the nitrogen of the amino acid fraction.

The asparagine isolated accounts for 55.6 per cent of the amide nitrogen of the amino acid fraction. Since any free aspartic acid in the plant juice should have been removed by the preliminary treatment with barium hydroxide and alcohol, the aspartic acid found in this fraction after hydrolysis probably represents asparagine which did not crystallize or which had been hydrolyzed by the

TABLE II.

Yields of Substances Isolated from the Amino Acid Fraction Obtained from Alfalfa Filtrate Representing 23.96 Kilos of the Fresh Plant.

Substance.	Amount.	N	Fresh plant.	Dry plant.	N as N of plant.	N as N of alfalfa filtrate.	Substance per gm. N of alfalfa filtrate.
	gm.	gm.	per cent	per cent	per cent	per cent	gm.
Asparagine.....	18.03	3.364	0.075	0.411	1.52	6.10	0.327
Aspartic acid.....	6.196	0.6524	0.026	0.112	0.294	1.18	0.112
Tyrosine.....	0.117	0.009	0.0005	0.0027	0.004	0.016	0.0021
Phenylalanine.....	0.821	0.0698	0.0034	0.019	0.032	0.127	0.015
Serine.....	2.407	0.321	0.010	0.055	0.144	0.582	0.0436
Leucine.....	2.07	0.221	0.0086	0.0472	0.095	0.401	0.0376
Valine.....	2.887	0.345	0.012	0.0658	0.155	0.625	0.0524
Alanine.....	1.163	0.183	0.0049	0.0265	0.082	0.332	0.021
Total.....	33.69	5.165	0.140	0.739	2.326	9.363	

acid solution during the removal of bases by means of phosphotungstic acid. Assuming this, the aspartic acid isolated is equivalent to asparagine containing an additional 21.5 per cent of amide nitrogen or 77.1 per cent in all. Whether or not an amide other than asparagine is present in this fraction is still undetermined, but with so large a part of the amide nitrogen of this fraction accounted for, it is evident that amides other than asparagine can occur only in relatively small proportion. Indications of the presence of a small amount of glutaminic acid were found after hydrolysis. This may mean that glutamine is also present, but conclusive evidence is lacking.

The presence of a considerable quantity of the hydroxyamino acid serine in the alfalfa plant is especially interesting. This acid has long been known as a constituent of proteins and there is no reason to believe that it is not widely distributed in plants; the difficulties presented by its isolation, however, are sufficient to explain why it has not previously been encountered in them. The use of the ester distillation method seems essential for its separation from complex mixtures. This method has been used in the analysis of amino acids from plant juices, so far as we are aware, only by Schulze and Winterstein (8) and they failed to obtain crystalline products from the residue from the distillation of the esters which would contain any serine present.

Alanine is also difficult to separate from complex mixtures unless the ester distillation method is employed. It is very soluble in water and, unless present in large proportion, would hardly be found by direct crystallization.

The presence of tyrosine, phenylalanine, leucine, and valine scarcely calls for comment except that it indicates the probable presence of most of the protein amino acids in alfalfa juice. Although plant physiologists have long considered that translocation of protein in the plant is effected by a decomposition of protein into asparagine and resynthesis of protein from this substance at another point (9), the presence of amino acids in appreciable amount in plant juices indicates that other, and perhaps more obvious, chemical reactions may play some part in the process. At the present time, however, the chief problem is strictly analytical. When we actually know what substances are present we shall be in a position to speculate upon the chemical reactions of metabolism. It is obvious from the meager results of this study of the alfalfa plant that we are still far from the solution of the problems presented by plant analysis.

EXPERIMENTAL.

Alfalfa filtrate, prepared from 23.96 kilos of fresh alfalfa¹ as described in a previous paper (7), was treated with saturated barium hydroxide solution until no further precipitate separated; this was removed and an equal volume of alcohol was added to the filtrate. The precipitate which

¹ The 23.96 kilos of fresh alfalfa employed contained 4387 gm. of dry material and 222 gm. of nitrogen.

formed was separated by centrifuging, and reagents were removed from the solution which was then concentrated and treated with mercuric acetate, sodium carbonate, and alcohol (Neuberg's reagent (6)) in the manner already described (1). The precipitate thus produced was removed, washed with 50 per cent alcohol, and decomposed with hydrogen sulfide. After removal of mercuric sulfide and concentration, the solution was treated with an excess of phosphotungstic acid in the usual way and the precipitate removed. The reagents were removed from the filtrate which was then concentrated *in vacuo* until crystalline material separated. The first crops consisted of plates which were apparently mixtures of amino acids and were later returned to the mother liquor. Subsequent crops consisted of nearly pure asparagine and a little tyrosine. These were separated by elutriation and recrystallized before weighing. The final crops were obtained by adding alcohol until a sirup separated and allowing the solution to stand several months. The yield of recrystallized asparagine was 18.03 gm. and of tyrosine, 0.117 gm. The asparagine contained 11.98 per cent of water of crystallization and 18.49 per cent of nitrogen; theory for $C_4H_8O_3N_2 \cdot H_2O$, H_2O , 12.00 per cent; N, 18.66 per cent. The tyrosine contained 7.6 per cent of nitrogen; theory, 7.74 per cent.

The indefinite mixtures of amino acids which separated first, and the mother liquors from recrystallizing the asparagine, were united with the main mother liquor which was then boiled with 20 per cent hydrochloric acid for 23 hours. The small amount of humin was filtered off on asbestos. The solution was concentrated to a sirup, alcohol added, and a small quantity of sodium chloride removed. An excess of crystalline barium hydroxide was added and the solution concentrated *in vacuo* at 50° to remove ammonia which was determined in the distillate. The amide nitrogen of the solution, less that of the asparagine separated, amounted to 1.344 gm. Since 1.661 gm. of ammonia nitrogen were found in the distillate, the ammonia nitrogen set free by secondary decomposition amounted to 0.317 gm. This is rather more than would be expected from a solution containing only simple amino acids and indicates that other and less stable substances were present.

The concentrated solution containing the barium salts of amino acids was treated with alcohol to make a concentration of 75 per cent and the precipitate, which contained the dibasic amino acids (10) removed after standing 18 hours. A further small precipitate was obtained by the addition of more alcohol.

The amount of nitrogen in each of the subfractions of the monoamino acid fraction is given in Table III.

Dibasic Amino Acids.—The precipitate was dissolved in dilute sulfuric acid, barium sulfate removed, and the excess sulfuric acid exactly removed with barium hydroxide. The solution was then boiled with excess of lead hydroxide (11) and allowed to stand overnight. The coarse, sandy precipitate was decomposed with sulfuric acid, all reagents were removed, and the solution was boiled with excess of copper hydroxide. A considerable quantity of copper aspartate readily separated on cooling the filtered solu-

662 Nitrogenous Constituents of Alfalfa. VI

tion. Further amounts of aspartic acid were obtained from the mother liquor by repeating the precipitation with lead and copper, but about one-third of the nitrogen of the original lead precipitate was obtained in final sirupy mother liquors from which nothing could be brought to separate. The yield of copper aspartate was equivalent to 6.196 gm. of aspartic acid. Dried at 130° it contained 32.56 per cent of copper; theory, 32.7 per cent.

The filtrate from the lead hydroxide precipitate was freed from lead, concentrated to a sirup, and saturated with hydrogen chloride. A trace of sodium chloride separated, followed by a small quantity of crystals which resembled glutaminic acid hydrochloride. Too little was obtained to identify. The sirupy mother liquor was freed from chloride with silver nitrate and a silver salt, insoluble at neutral reaction to litmus, precipitated by alternate additions of silver nitrate and sodium hydroxide. The free acid was obtained as a white crystalline powder which did not melt below 315°.

TABLE III.

Distribution of Nitrogen in the Amino Acid Fraction from 23.96 Kilos of Alfalfa.

	Nitrogen.
	gm.
Asparagine.....	3.364
Tyrosine.....	0.090
Ammonia.....	1.661
Dibasic amino acid fraction.....	3.040
Monoamino acid fraction.....	3.588
Loss in humin, etc.....	0.720
Total.....	12.463

It was sparingly soluble in cold water when pure and could be recrystallized from this solvent. It contained 23.8 per cent of nitrogen, none of which was amino nitrogen, and gave a positive murexide test and a silver salt soluble in excess of ammonia. The properties of this substance recall those of uracil but its aqueous solution was faintly acid to Congo paper and Wheeler and Johnson's color test (12) was negative. Only a small amount was obtained and further work on its identity is, for the present, postponed.

Monoamino Acids.—The filtrate from the barium hydroxide and alcohol precipitate was freed from reagents, concentrated to a sirup, and the amino acids were esterified by distilling absolute alcohol containing hydrogen chloride through the sirup maintained at 105°. No glyocoll ester hydrochloride separated on allowing the esters to stand overnight. The ester hydrochlorides were dissolved in chloroform and the esters liberated by means of anhydrous baryta (13). The chloroform was removed by distillation at atmospheric pressure and the esters were distilled at 18 mm. up to a vapor temperature of 96°. The distilled esters were hydrolyzed by

boiling with water and the solution was concentrated to dryness. The dry amino acids were extracted with absolute alcohol to remove proline and separated by fractional crystallization into nine fractions, the final crops from dilute alcoholic mother liquors. The first fraction contained 10.81 per cent of nitrogen and was practically pure leucine; theory, 10.69 per cent. The fifth fraction contained 11.96 per cent of nitrogen and was apparently wholly valine; theory, 11.96 per cent, while the intermediate fractions were mixtures of these two amino acids. The sixth crop showed a marked rise in nitrogen content to 13.8 per cent and the final crops contained nitrogen indicating that they were mixtures of valine and alanine in approximately equal proportions. From the weight and nitrogen content of these fractions, the 6.12 gm. of dry amino acids obtained were calculated to consist of 2.07 gm. of leucine, 2.89 gm. of valine, and 1.16 gm. of alanine. The mixtures of valine and alanine were converted to copper salts, valine copper was removed by crystallization from water, and alanine recovered from the mother liquor. The valine copper contained 21.52 per cent of copper; theory, 21.49 per cent. The alanine yielded an α -naphthylhydantoic acid derivative melting at 202.5°C. and containing 10.8 per cent of nitrogen; theory, 10.85 per cent. Neuberg and Rosenberg give the melting point of this compound at 202° (14).

Absolute alcohol extracted little more than a trace of material from the dry amino acids. The extract was evaporated to a few drops of sirup which yielded a copper salt soluble in absolute alcohol. Although this is an indication that proline was present, the quantity was too small for identification.

The distillation residue was dissolved in water and extracted with ether. The ester extracted by ether was hydrolyzed with hydrochloric acid and 1.0 gm. of phenylalanine hydrochloride was obtained by crystallization. The free amino acid was obtained in the usual way and contained 8.58 per cent of nitrogen; theory, 8.49 per cent.

The water-soluble ester was hydrolyzed with barium hydroxide, reagents were removed, and the solution was concentrated to a sirup which slowly crystallized to a waxy mass. A portion of this was weighed out and converted to the α -naphthylhydantoic acid derivative. This melted at 192° and contained 10.22 per cent of nitrogen; theory for the derivative from serine, 10.20 per cent. Neuberg and Rosenberg give the melting point of this compound at 192° (14). Assuming an 89 per cent yield of the derivative as obtained by these authors, the waxy mass contained 62 per cent of serine, representing a yield of 2.41 gm.

SUMMARY.

A fraction containing asparagine and the free amino acids of the juice of the alfalfa plant has been obtained by methods recently described (1) and examined by the methods of protein analysis. Asparagine, aspartic acid, tyrosine, phenylalanine,

serine, leucine, valine, and alanine were isolated. These substances make up at least 29.5 per cent of the solids of the fraction and, together with the ammonia set free by hydrolysis, 54.8 per cent of the nitrogen.

The aspartic acid obtained after hydrolysis probably represents asparagine. At least 55 per cent of the amide nitrogen of the amino acid fraction is accounted for as asparagine and the aspartic acid found after hydrolysis indicates that more of this substance is present. Although no other amide has yet been found, it is probable that other substances yielding ammonia on mild acid hydrolysis are present in the juice of the alfalfa plant.

Serine and alanine, so far as we can learn, have not previously been reported as occurring in plant juices. They were definitely identified as α -naphthylhydantoic acid derivatives.

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ENERGY EXPENDITURE OF WOMEN DURING HORIZONTAL WALKING AT DIFFERENT SPEEDS.

BY H. MONMOUTH SMITH AND DORTHA BAILEY DOOLITTLE.

From the Chemical Laboratory, Massachusetts Institute of Technology, Cambridge.)

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Measurements of the energy expenditure of men during horizontal walking have been made by a number of investigators, a summary of which has been given by Benedict and Murschhauser¹ and Smith² but the study of the energy expenditure of women during walking is limited. Durig³ found with one subject (Frau D.) walking at a speed of 65 meters per minute in a hall in Vienna carrying a load of 16.5 kilos an expenditure of 0.604 m. calorie per kilo per meter moved and with the same subject on rough path at a high altitude on the Spornier Alp at a speed of 72 meters per minute an expenditure of 0.668 gm. calorie per kilo per meter moved. These values were somewhat larger than those found by Durig on himself under similar conditions. Durig was interested primarily in the effect of altitude upon the energy expenditure during walking and there appears to be no systematic study of the energy expenditure of women *per se*.

Benedict and Johnson⁴ in an experiment with a group of 25 young women walking in a chamber at the slow rate of 1.08 miles per hour found an expenditure of 62 calories per mile in excess of the sitting requirement and a total expenditure of 2.44 calories per kilo of body weight per hour. The expenditure over the sitting requirement was 1.20 calories per kilo per hour. This is equivalent to 0.71 gm. calorie per kilo of body weight moved 1

¹ Benedict, F. G., and Murschhauser, H., *Carnegie Institution of Washington, Pub. No. 231*, 1915.

² Smith, H. M., *Carnegie Institution of Washington, Pub. No. 309*, 1922.

³ Durig, A., *Arch. ges. Physiol.*, 1906, cxiii, 213.

⁴ Benedict, F. G., and Johnson, A., *Proc. Am. Phil. Soc.*, 1919, lviii, 96.

meter on the horizontal walking at the rate of 29 meters per minute.

The object of our study was to determine the energy expended by a group of women when walking on a level; it was also planned to so select the speeds of walking that the effect, if any, of slow walking might become apparent.

Since the total energy expended in level walking consists of the requirements for simple body maintenance in a position of quiescence plus the superimposed requirements for the muscular movements of walking it is necessary first to determine this resting requirement. The position selected for the base line of this study was that of standing quietly without support, as this seemed a more natural point from which to compute the superimposed effects of walking *per se*. The study therefore consists of a series of determinations of the standing metabolism besides those for walking.

The increase in the heat output of walking over that for standing is naturally dependent upon the weight of the subject and the speed at which she walked, and as a basis for comparison the increase in the energy expended in walking over that required for standing has been computed per horizontal kilogrammeter, *i.e.* 1 kilo of body weight moved 1 meter on a horizontal plane.

Methods of Measurement.

The method selected for collecting the respiratory gases was the well known one employed by Douglas⁵ in which the subject wears on the back a light rubber bag of approximately 100 liters capacity connected with a mouthpiece or mask of some sort. Our subjects used a half-mask of the Siebe-Gorman type thus avoiding the nose-clip and any possible interference with normal respiration due to the presence of the mouthpiece. The tightness of the mask over the face was tested at each experiment by closing the end of the exit tube with the palm of the hand while the subject exerted pressure upon the system by the lungs. The expired gases collected in the bag were measured by passing them through an integrating meter capable of showing differences of 10 cc. The meter had been calibrated⁶ by passing weighed quantities of oxygen

⁵ Douglas, C. G., *J. Physiol.*, 1911, xlii, p. xvii.

⁶ Benedict, F. G., *Phys. Rev.*, 1906, xxii, 294.

through it and all measurements were corrected for temperature and pressure. Two samples of air were withdrawn into the Haldane gas samplers during the transfer of the air from the bag through the meter and analyzed on a Haldane⁷ gas apparatus for carbon dioxide and oxygen. The subjects walked on a cement floor in a straight corridor of the Institute which was fairly free of use as a thoroughfare and which was open at either end to the outside air. Two convenient points 81.7 meters distant were marked on the floor and each exercise consisted of walking up and down this distance twice or a total of 326.8 meters except at the slowest speed when but one complete turn was made. In addition each exercise was immediately preceded by a preliminary walk once up and back. Three speeds were selected of 30, 60, and 90 meters a minute, which after a little experience, could be very closely maintained by the pace maker who walked by the subject's side with the stop-watch and turned the valves on the respiration apparatus as the subject crossed the marks.

Routine of Experiment.

The subject came to the laboratory between 7.30 and 8.00 a.m. in the post-absorptive condition and after a few minutes of rest the bag and mask were adjusted and tested for tightness. She then stood quietly preliminary to the standing experiment during which time the radial pulse was frequently noted as an evidence that conditions were normal. After approximately 10 minutes of preliminary standing the valve connecting with the bag was turned and the standing period proper began, this lasted from 8 to 10 minutes during which time the reading of the pulse was continued and two samples of the room air were collected for analysis.

At the close of the standing period the bag was replaced by another, the apparatus tested for tightness, and the subject was weighed as thus equipped for walking. The subject and assistant then proceeded to the corridor just outside the laboratory door and the walking period began after the preliminary walk. The second walking period followed as soon after the first period as a fresh bag could be adjusted. During the periods of walking two samples of the corridor air were collected for analysis. The composition of this air was very uniform from day to day due to the good ventilation of the hall and averaged 0.05 per cent for carbon dioxide and 20.87 per cent for oxygen. At the end of the second walking period the subject was again weighed after which the apparatus was removed and breakfast was served to her. At this time data were collected as to her

⁷ Haldane, J. S., *Methods of air analysis*, London, 2nd edition, 1918.

previous meal, activities, and clothes worn from which her body weight was computed.

The respiratory gases collected in the Douglas bags were measured as soon as the periods ended and two samples were withdrawn from each for analysis at the time of the measurement. An analysis of each gas sample was made and in case of any non-agreement within the limits of accuracy of the apparatus, the analysis was repeated. The average of the analyses was taken for computation. As a check upon the efficiency and accuracy of the apparatus an analysis of outdoor air was made daily as suggested by Haldane.⁸ Agreement with the accepted composition of outdoor air of 20.94 per cent oxygen was accepted as indicating the reliability of the apparatus.

Subjects.

The subjects of the experiments were young women, all but one of whom were students at the Massachusetts Institute of Technology and all were in good health and spirits. They all had an interest in the work and cooperated intelligently. The principal data regarding the subjects are summarized in Table I.

The body weight used in computing the body area was obtained by deducting the estimated weight of the clothing worn at the time the subject was weighed. The estimation of the weight of the clothing was made by obtaining the average weight of similar pieces of women's apparel.

Statistics of Experiments.

The results of the standing experiments for each subject have been averaged and are given in Table II. The results are in each case the averages of nine or ten experiments.

The respiratory quotients indicate normal conditions and the experiments from which these figures are the averages were quite uniform. E. L. F. had an average respiratory quotient of 0.86, a value somewhat higher than that for the other subjects yet seven out of the ten experiments with her were within 0.02 of this value and in all ten of these experiments she showed a higher quotient

⁸ Haldane, J. S., *Methods of gas analysis*, London, 2nd edition, 1918.

than did the other subjects. The computed heat values are obtained from the oxygen consumption and its calorific value according to Zuntz.⁹ We have made use of Carpenter's tables¹⁰ for this purpose. The total heat thus computed is also given as per kilo of body weight per hour and as per square meter of body surface per hour. For the body weights there have been taken the averages of the weights on the days of the standing experiments.

TABLE I.

Age, Weight, Height, and Body Area of Subjects Used in Experiments.

Subject.	Age.	Weight.	Height.	Body area.*	Remarks.
	<i>yrs.</i>	<i>kg.</i>	<i>cm.</i>	<i>sq. m.</i>	
D. B. B.	26	45.42	158.6	1.43	Graduate, active, quick in movements.
E. P. C.	19	47.24	152.3	1.41	Active, well developed, calisthenics daily.
F. H. C.	24	50.27	151.5	1.44	Graduate, interested in outdoor sports, active.
R. E. D.	30	58.81	159.9	1.61	Slow in movements.
G. M. F.	21	51.39	156.7	1.48	Quick in movements.
E. L. F.	18	48.41	157.3	1.46	Nervous, excitable.
F. J. M.	30	60.19	162.1	1.65	Graduate, well developed, calisthenics daily.
B. S. W.	20	55.21	158.2	1.55	Graduate, heavy build, strict vegetarian.†
G. W.	25	48.52	159.4	1.48	Teacher, active, nervous, athletic.

* According to Du Bois' height-weight chart.

† B. S. W. though anxious to cooperate was not a very satisfactory subject. She was unable to do the standing test easily and became faint on several occasions.

The body surface is that computed from the weights given in Table I.

The average heat output per kilo of body weight per hour is 1.07 calories or 36.4 calories per square meter of body surface per hour.

⁹ Zuntz, N., and Schumburg, W. A. E. F., *Physiologie des Marsches*, Berlin, 1901, 361.

¹⁰ Carpenter, T. M., *Carnegie Institution of Washington, Pub. No: 303*, 1921, 104.

Walking Experiments.

The results of the walking experiments which followed the standing periods are collected in Table III. Since the heat values have been calculated from the oxygen consumption and as this value is most liable to error in the experimental work the range in the oxygen consumption for the different experiments has been inspected. These values show a maximum variation up to 8 per cent but with an approximate variation of 3 per cent from the

TABLE II.

Metabolism of Subjects during Standing in Experiments without Food.
(Values per Minute.)

Subject.	No. of experiments.	Pulmonary ventilation (reduced).	Average pulse rate.	Carbon dioxide.	Oxygen.	Respiratory quotient.	Heat computed.		
							Total per minute.	Per kilo of body weight per hour.	Persq. m. body surface per hour.
		<i>liters</i>		<i>cc.</i>	<i>cc.</i>		<i>cals.</i>	<i>cals.</i>	<i>cals.</i>
D. B. B.	10	5.93	95	153	188	0.81	0.91	1.20	37.8
E. P. C.	10	4.52	94	144	172	0.83	0.84	1.07	35.3
F. H. C.	9	4.42	88	144	173	0.83	0.82	1.02	35.5
R. E. D.	10	6.88	93	178	218	0.82	1.05	1.07	39.2
G. M. F.	10	5.84	96	157	186	0.84	0.90	1.05	36.4
E. L. F.	10	4.87	97	154	175	0.86	0.87	1.08	35.7
F. J. M.	10	5.38	95	153	188	0.81	0.91	0.91	33.1
B. S. W.	9	5.45	89	166	201	0.82	0.98	1.06	37.7
G. W.	9	5.19	90	155	194	0.80	0.93	1.15	37.7
Average.....		5.39		156	188		0.91	1.07	36.4

average. There is therefore a probable error in the neighborhood of 5 per cent. It is noted that the average speed of walking was very closely maintained at the rate of 30, 60, and 90 meters a minute. The average reduced ventilation for the nine subjects at these speeds as derived from this table was 8.48, 11.22, and 15.94 liters per minute or an increase over the standing requirement of 57, 108, and 196 per cent. Similarly the increase in the oxygen requirements was 100, 188, and 318 per cent. The average total

TABLE III.

Metabolism of Subjects during Horizontal Walking in Experiments without Food. (Values per Minute.)

Subject.	No. of experiments.	Distance walked.	Pulmonary ventilation (reduced).	Carbon dioxide.	Oxygen.	Respiratory quotient.	Heat computed.		
							Total per minute.	Per kilo of body weight per hour.	Per sq. m. body surface per hour.
		meters	liters	cc.	cc.		cals.	cals.	cals.
D. B. B.	6	30.0	9.50	291	364	0.78	1.77	2.32	74.4
	6	60.1	10.91	396	507	0.80	2.43	3.19	102.0
	6	89.5	15.06	542	708	0.77	3.37	4.49	141.6
E. P. C.	8	30.2	7.37	291	354	0.82	1.71	2.18	72.0
	8	59.5	8.62	364	473	0.77	2.25	2.87	95.4
	4	90.2	14.35	612	754	0.81	3.63	4.59	153.6
F. H. C.	8	30.1	7.60	297	360	0.83	1.74	2.08	72.0
	6	59.8	9.27	370	467	0.79	2.24	2.66	92.4
	6	90.4	13.83	573	714	0.80	3.43	4.10	141.6
R. E. D.	6	30.0	9.88	310	398	0.78	1.90	1.94	70.8
	8	60.1	13.42	448	594	0.81	2.86	2.90	106.8
	6	90.1	20.02	691	856	0.81	4.11	4.23	154.2
G. M. F.	6	29.9	8.67	301	377	0.80	1.81	2.11	73.2
	6	60.1	12.70	431	521	0.83	2.52	2.97	102.0
	6	90.3	15.89	571	702	0.81	3.38	3.94	136.2
E. L. F.	8	29.9	8.77	312	378	0.83	1.82	2.26	75.0
	6	60.0	12.40	467	554	0.83	2.68	3.32	110.4
	6	90.2	16.40	668	779	0.86	3.79	4.69	156.0
F. J. M.	8	30.0	8.48	316	392	0.80	1.88	1.87	68.4
	6	60.0	11.32	460	594	0.77	2.83	2.80	103.2
	6	90.1	15.89	666	872	0.76	4.14	4.16	150.6
B. S. W.	6	30.0	8.31	305	390	0.78	1.86	2.01	72.0
	12	60.1	11.17	433	557	0.78	2.65	2.89	102.6
	5	90.7	16.47	654	819	0.79	3.94	4.30	152.4
G. W.	6	30.0	7.75	296	382	0.78	1.82	2.26	73.8
	6	60.0	11.22	463	593	0.78	2.83	3.47	114.6
	6	90.1	15.52	662	856	0.77	4.07	5.04	165.0

heat expended per minute for the nine subjects was 1.81, 2.59, and 3.76 calories for the three speeds equivalent to 2.11, 3.01, and 4.39 calories per kilo of body weight per hour or 72.4, 103.3, and 150.1 calories per square meter of body surface per hour.

The average heat expenditure above the standing requirement was 1.05, 1.95, and 3.33 calories per kilo of body weight per hour or 99, 184, and 315 per cent for the three speeds respectively.

The close agreement in the increase in the oxygen consumed and the energy expended due to a practically stationary respiratory quotient, indicates that for cases of moderate exercise such as here employed, an assumed respiratory quotient of 0.80 might be taken and the heat values might be calculated from this without introducing any serious error.

From the averages given in Table II for standing and in Table III for the energy expended during walking the increase in the metabolism over the standing requirements is computed and given in Table IV.

In this table the value used for the standing requirements of the subject in computing the increase due to walking is not the average of all of her standing experiments but only for those days when the particular speed under consideration was carried out. The distance walked multiplied by the body weight plus the weight of the mask, bag, etc., gives the number of horizontal kilogrammeters, *i.e.* the number of kilos moved 1 meter on a horizontal, and the increase in the heat output over the standing value divided by the number of horizontal kilogrammeters gives the energy expended per horizontal kilogrammeter, shown in the last column. The average of this value for the nine subjects for the speeds of 30, 60, and 90 meters per minute is 0.528, 0.486, and 0.553 gm. calorie respectively. Smith¹¹ gives 0.449, 0.463, and 0.553 gm. calorie as the averages of some walking experiments with E. D. B. on a treadmill for speeds of 37, 65, and 91 meters per minute and Benedict and Murschhauser¹ report 0.498 gm. calorie for their Subject II at speeds of 60 to 65 meters per minute and 0.524 gm. calorie for speeds between 90 and 100 meters per minute. Although the speeds for these different groups do not exactly coincide the differences are not such as to indicate that the energy expended by

¹¹ Smith,² p. 149.

TABLE IV.

Increase in the Heat Output of Subjects during Horizontal Walking in Experiments without Food. (Values per Minute.)

Subject.	No. of experiments.		Weight as walked.	Distance walked.	Horizontal kilogram-meter.	Total heat.		Increment in heat above standing value.	
	Standing.	Walking.				Standing.	Walking.	Total.	Per horizontal kilogram-meter
			kg.	meters		cals.	cals.	cals.	gm. cal.
D. B. B.	3	6	51.16	30.0	1535	0.91	1.77	0.86	0.560
	3	6	51.15	60.1	3074	0.91	2.43	1.52	0.495
	3	6	50.48	89.5	4518	0.90	3.37	2.47	0.547
E. P. C.	4	8	53.43	30.2	1614	0.82	1.71	0.89	0.551
	4	8	53.65	59.5	3192	0.82	2.25	1.43	0.448
	2	4	53.87	90.2	4859	0.90	3.63	2.73	0.562
F. H. C.	3	6	55.28	30.1	1664	0.81	1.74	0.93	0.559
	2	6	55.59	59.8	3324	0.82	2.24	1.42	0.427
	3	6	55.34	90.4	5002	0.86	3.43	2.57	0.514
R. E. D.	3	6	64.90	30.0	1947	1.08	1.90	0.82	0.421
	4	8	65.29	60.1	3924	1.04	2.86	1.82	0.464
	3	6	64.49	90.1	5811	1.03	4.11	3.08	0.530
G. M. F.	3	6	57.12	29.9	1708	0.87	1.81	0.94	0.550
	3	6	56.61	60.1	3402	0.96	2.52	1.56	0.459
	3	6	57.19	90.3	5165	0.88	3.38	2.50	0.484
E. L. F.	4	8	54.02	29.9	1615	0.89	1.82	0.93	0.576
	3	6	53.99	60.0	3239	0.88	2.68	1.80	0.556
	3	6	54.09	90.2	4879	0.83	3.79	2.96	0.607
F. J. M.	4	8	65.70	30.0	1971	0.90	1.88	0.98	0.497
	3	6	66.35	60.0	3980	0.91	2.83	1.92	0.482
	3	6	65.26	90.1	5881	0.91	4.14	3.23	0.549
B. S. W.	3	6	60.77	30.0	1823	0.97	1.86	0.89	0.488
	9	12	60.41	60.1	3631	0.98	2.65	1.67	0.460
	3	5	60.46	90.7	5484	0.97	3.94	2.97	0.542
G. W.	3	6	53.88	30.0	1616	0.91	1.82	0.91	0.563
	3	6	54.51	60.0	3271	0.93	2.83	1.90	0.581
	3	6	54.13	90.1	4877	0.94	4.07	3.13	0.642

the women in walking is different from that of the men. The figures also show that the energy expenditure per horizontal kilogrammeter of the women like that for the subject E. D. B. of

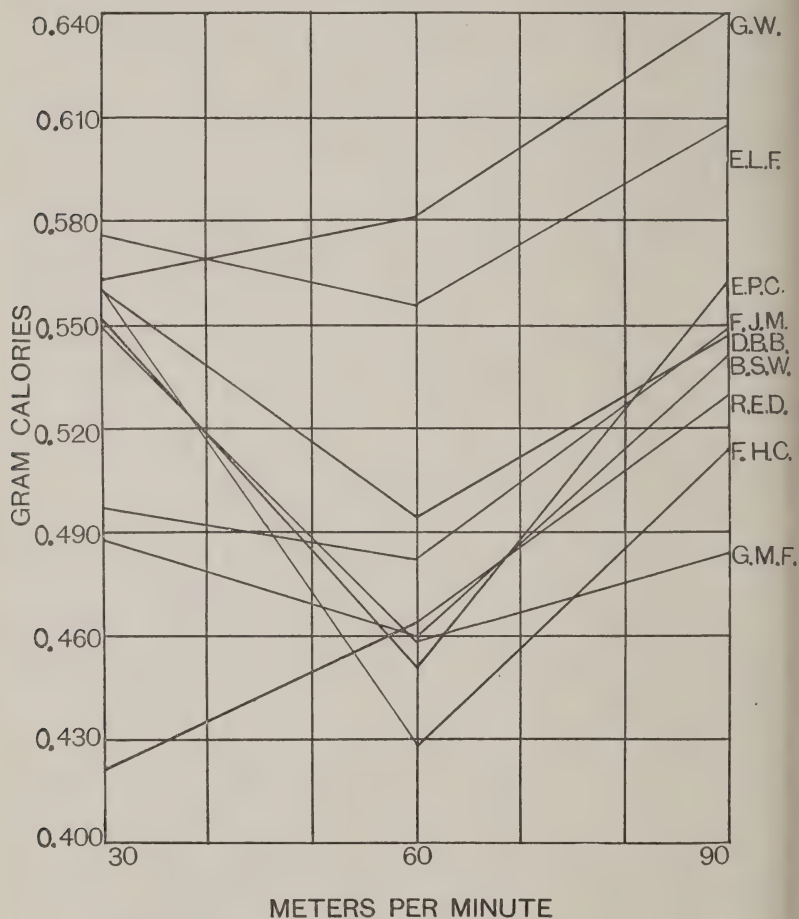


FIG. 1. Energy expenditure per horizontal kilogrammeter at speeds of 30, 60, and 90 meters per minute.

Smith is usually less at the normal speed of 60 meters a minute than for the slow rate of 30 meters or the faster rate of 90 meters per minute.

The relation between the speed and the energy output per hori-

zontal kilogrammeter from our results is graphically shown in Fig. 1 where the energy output for each of the nine women at the three speeds has been plotted. The curves show that with the two exceptions of R. E. D. and G. W. the energy expenditure was greater at 30 than at 60 meters per minute and that in three cases the expenditure was larger at 30 than at 90 meters. Most of the subjects found the slow walking tiresome but R. E. D. whose energy was lowest at 30 meters said she "liked the slow rate, it rested her."

TABLE V.

Comparison of the Oxygen Consumption during the Menstrual Period of Subjects in the Standing Position. (Values per Minute.)

Subject.	O ₂ consumption.		Increase in O ₂ consumption above average.	
	Average of non-menstrual days.	Menstrual period.		
	cc.	cc.	cc.	per cent
D. B. B.	188	193	+5	+2.66
F. H. C.	173	174	+1	+0.58
G. M. F.	187	184	-3	-1.60
E. L. F.	178	185	+7	+3.93
F. J. M.	188	191	+3	+1.60
	188	186	-2	-1.06
B. S. W.	204	194	-10	-4.90
	204	221	+17	+8.34
	204	200	-4	-1.95
G. W.	193	204	+11	+5.70

During this study there were 10 days when the subjects were in the menstrual period. The standing metabolism as measured by the oxygen consumption on these days and compared with the average oxygen consumption on the non-menstrual days of the same subject is shown in Table V. As seen in the table seven subjects are included in this data, five on 1 day only, one on 2 days of the same period and on 1 day a month later, and one on 2 days of the same period. Four subjects showed an increase in the metabolism, one a decrease, while of those on whom data were obtained on more than 1 day both increases and decreases were found. The data here shown were all within the menstrual period but in what stage of the period is not known. Wakeham¹² claims

¹² Wakeham, G., *J. Biol. Chem.*, 1923, lvi, 555.

that the basal metabolism is subject to a premenstrual rise and a distinct fall during or immediately after menstruation. As our data were within the menstrual period we are not able to submit any evidence on this point. Our evidence is rather in support of the conclusions of Blunt and Dye¹³ who found that the lying metabolism was unaffected by the menstrual period.

SUMMARY.

The energy expenditure of women during horizontal walking is of the same degree as that of men when walking at moderate speeds, this value is from 0.450 to 0.550 gm. calorie per horizontal kilogrammeter.

The energy expenditure per horizontal kilogrammeter is greater at an abnormally slow speed than at moderate speeds.

The menstrual period appears to be without definite influence on the standing metabolism.

The authors wish to acknowledge the aid of the Ellen H. Richards Memorial Research Fund of the Massachusetts Institute of Technology in carrying out this work.

¹³ Blunt, K., and Dye, M., *J. Biol. Chem.*, 1921, xlvii, 69.

THE COLORIMETRIC pH TEST OF WATER OR UNBUFFERED SOLUTIONS.

BY HANS T. STERN.

(From the Plaut Research Laboratory of Lehn and Fink, Inc., Bloomfield.)

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INTRODUCTION.

In many cases of routine control of the H ion concentration within the neutral range, the only feasible test is the one by means of indicators. This is especially the case when a long series of measurements has to be carried out or when substances are present which prevent the electrode from responding to the H ions. The well known difficulties encountered in testing unbuffered solutions with the hydrogen electrode have lately been explained and apparently overcome by Beans and Hammett.¹ Unfortunately their electrode becomes inactive very soon and involves many difficulties. For unbuffered solutions, no other electrode seems to have been perfected that is satisfactory. On the other hand, the colorimetric method is open to two principal objections. The salt and protein errors of the indicators can be taken into account by adequate methods.² However, variations occur with the different dye products which are difficult to explain.

An investigation interesting in this respect is the comparison made by Ramann and Sallinger³ between the indicator series of Clark and Lubs and of Michaelis (nitrophenols). They find quite frequently differences of 0.1 to 0.2 pH. This may be due mainly to salt error, because buffer solutions had to be used. It seems however hardly possible that this factor should account for the whole amount.

¹ Beans, H. T., and Hammett, L. P., *J. Am. Chem. Soc.*, 1925, xlvii, 1215.

² Kolthoff, I. M., *Rec. trav. chim.*, 1925, xlv, 275. Ramage, W. D., and Miller, R. C., *J. Am. Chem. Soc.*, 1925, xlvii, 1230. Hirsch, E. F., *J. Biol. Chem.*, 1925, lxxiii, 55.

³ Ramann, E., and Sallinger, H., *Z. anal. Chem.*, 1923, lxxiii, 292.

In buffered solutions only the varying color strengths come into effect and these may again be eliminated by preparing standards with the same dye. In unbuffered solutions there is, in addition to this, a marked effect noticeable due to the varying alkali content of the dye product and possibly to variations in the dye constitution. Many investigators also ascribe much influence to the carbon dioxide content of the air.

Experiments with Bromthymol Blue and Para-Nitrophenol.

Different samples of bromthymol blue gave the following pH values in the same sample of laboratory distilled water.

	pH
Bromthymol blue solution from laboratory stock.....	6.4
Solution from LaMotte bromthymol blue.....	6.0
Same, with alkali according to W. M. Clark.....	6.7
Other sample from LaMotte bromthymol blue.....	6.2
Bromthymol blue solution from Hynson, Westcott, and Dunning.....	5.8

The tests were carried out by taking great care to eliminate the influence of the atmosphere and of the wall of the container. Carefully cleaned tubes of Nonsol glass with narrow necks and bearing a mark at 10 cc. were used. Before each test a tube was rinsed out with the water to be tested, then 0.6 cc. of the 0.04 per cent indicator solution was pipetted into both the water and the control tube with buffer solution. This control has to be prepared every time, for the percentage of the dye is not a sufficient indication of its color strength, and because standard tubes do not keep indefinitely.

When alkali was not used in dissolving the dye, the dye was first dissolved in pure alcohol and then diluted with distilled water. Later on it was found advantageous always to dissolve the dye first in alcohol and then add a quantity of alkali necessary to obtain a neutral indicator solution. In preparing different solutions of the same indicator without alkali, it is readily seen from their color that their acidity varies within wide limits. A solution of pure, recrystallized bromthymol blue from Hynson, Westcott, and Dunning had the color of a potassium bichromate solution. These solutions must, therefore, induce a varying acidity in unbuffered solutions, into which they are brought for testing.

Michaelis and Krüger⁴ have determined the "acid error" of para-nitrophenol and have given it a thorough theoretical consideration assuming, of course, that the dye which they were using was chemically pure. They also give a procedure for testing tap water. According to these authors bromthymol blue being used in a much lower concentration on account of its high color strength, should not show any noticeable influence from the acidity of the dye.

It appears possible, but not practical, to determine the source of the variations mentioned, through an exact analysis of the dye. The author preferred to use conductivity water for testing the different bromthymol blue products and to adjust the indicator solutions by means of the proper amounts of alkali to show a neutral reaction in this water. The salt error may then be estimated by addition of highly purified neutral salts. To carry out this investigation successfully the preparation of a water of high purity was necessary.

Conductivity water was prepared by first distilling in an insoluble glass container (Nonsol or Jena) under reduced pressure from $\text{KMnO}_4 + \text{H}_2\text{SO}_4$ and afterwards from $\text{Ba}(\text{OH})_2$. A stream of CO_2 -free air was constantly passed through the water and the entire apparatus. The distillate was kept in a 400 cc. steamed Nonsol flask closed by a stopper with a syphon tube and a tube with soda lime as described by Arndt.⁵ The electrical conductivity of the water was determined, and the water always used on the same day in which it was prepared. The batch used for the following experiments had an electrical conductivity of 0.7×10^{-6} mhos.

Two different bromthymol blue solutions were prepared in the way described above. The pH of the indicator solutions was determined electrometrically and compared with different solutions of 0.04 per cent concentration.

	pH
A solution of pure, recrystallized bromthymol blue, giving a strongly acid reaction in conductivity water.....	3.17
The same solution, adjusted.....	7.36

⁴ Michaelis, L., and Krüger, R., *Biochem. Z.*, 1921, cxix, 317-321.

⁵ Arndt, K., *Handbuch der physikalisch-chemischen Technik*, Stuttgart, 2nd edition, 1923, 651.

Another adjusted bromthymol blue solution.....	7.35
A bromthymol blue solution showing an alkaline reaction in conductivity water.....	7.76

The color of the adjusted bromthymol blue solutions is a dark green without red translucence. With more alkali it becomes rapidly blue. Unfortunately this solution is not stable. Both solutions, though kept in paraffined Nonsol flasks showed a red translucence after a period of about 2 months and gave with conductivity water a pH of 6.4. The pH determined electrometrically was then found to be 6.16 to 6.38. The reading varied within about 10 millivolts. One sample kept in a bottle of ordinary glass turned to a blue color.

From these facts, it can be seen that bromthymol blue in solution undergoes changes which so far as we can determine, are uncontrollable. A further indication in this direction will be found in the last part of this article and in the publication of Clark and Lubs⁶ on this indicator.

Another indicator, covering approximately the same pH range is para-nitrophenol. On account of its relatively simple constitution this indicator is not likely to undergo any changes similar to those found in bromthymol blue. To obtain a solution of such an alkalinity so that it will show a neutral reaction in pure water, the following procedure was adopted:

5.00 cc. of the 0.3 per cent para-nitrophenol solution in pure water was titrated with 1/10 N KOH using Biilmann's quinhydrone electrode as an indicator of the H ion concentration. 0.33 to 0.34 cc. 1/10 N KOH was necessary to bring it to pH 7, the beginning P.D. corresponding to pH 4.90. From this the amount of alkali necessary for the whole batch was calculated. The amount of alkali used corresponds to approximately 1/3 mol of the indicator concentration (assuming the dye powder to be 100 per cent pure). A test made then with conductivity water gave a neutral reaction.

Influence of the Atmosphere.

When tests in unbuffered solutions are made with bromthymol blue there is always a noticeable change to a more acid reaction from 2 to 10 minutes after the indicator has been added. This is mostly ascribed to absorption of CO₂ from the air⁷ because the

⁶ Clark, W. M., and Lubs, H. A., *J. Washington Acad. Sc.*, 1916, vi, 486.

⁷ Dawson, L. E., *J. Phys. Chem.*, 1925, xxix, 551.

change does not occur in buffer solution. Certain experiments seemed to indicate otherwise. The color change in question occurred as readily with distilled water which had been standing in contact with the atmosphere for a long time. Conductivity water gave the same initial pH value after standing exposed to the air for 1 hour as when freshly drawn from the container. Moreover para-nitrophenol did not give a noticeable change towards the acid side within 10 minutes and the next day showed a slightly more alkaline reaction.

Fales and Nelson⁸ found that with para-nitrophenol indicator their laboratory distilled water showed a pH of 6.2 and after redistillation 5.8. Elimination of CO₂ could only have increased the pH value. It is most probable that the first value should be above 7. The loss might then be explained by elimination of NH₃.

SUMMARY.

1. A bromthymol blue solution for testing unbuffered solutions may be satisfactorily prepared by adding alkali until a pure dark green color is obtained.
2. Para-nitrophenol is recommended as a more reliable indicator for unbuffered solutions when it is used in a neutral solution.
3. Carbon dioxide from the atmosphere does not seem to affect the colorimetric pH test.

⁸ Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2769.

THE MUCOPROTEINS OF THE SNAILS, *HELIX ASPERSA* AND *HELIX POMATIA*.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The problem of the structure of complex proteins containing carbohydrates in their molecule is still a subject of discussion. The fundamental questions which have been debated during the last decades and which have been the subject of many controversies are concerned with the nature of the carbohydrate group present in the molecule of these substances. Since the discovery of mucoproteins or mucins, as all of them were termed originally, three mutually antagonistic views were advanced. Chronologically, they were the following: All mucoproteins are physical mixtures of a polysaccharide, resembling dextrin, and proteins. The polysaccharide was termed "animal gum" (Landwehr¹). Through the experimental criticism of Hammarsten,² this view was modified by its author and in its final form it assumed a chemical union between the animal gum and the protein. The second view, of more recent date, was that the carbohydrate complexes of all mucoproteins were conjugated sulfuric acids of the type of mucioitin sulfuric acid. The third theory presented the structure of this group of substances in the form of a peptide or glucosidic linking between simple sugars (amino sugars) and proteins.

There were authorities who believed in the existence of representatives of each one of the three types of substances. Theoretically, every one of the three theories is possible and, therefore, the final decision will depend only on facts. However, the number of complex proteins containing carbohydrates in their molecules is very great and it will require much time and labor to analyze all of them.

¹ Landwehr, H. A., *Z. physiol. Chem.*, 1882, vi, 74.

² Hammarsten, O., *Arch. ges. Physiol.*, 1885, xxxvi, 412.

A very critical and, for its time, a classical investigation into the question of mucoproteins was carried out by Hammarsten 40 years ago. Hammarsten came to the conclusion that two types of proteins containing carbohydrates in their molecules were obtainable from *Helix pomatia*. One type is represented by the substances obtainable from the mucus secreted by these animals and by that extracted from the foot of the animal. These mucoproteins are characterized by their elementary composition which does not differ much from that of simple proteins. In distinction from these mucoproteins, he prepared one from the "protein glands" of these animals. The latter substance possessed a peculiarly low nitrogen content varying between 6 and 8 per cent, and readily yielded on extraction with water or better on extraction with dilute alkali, a polysaccharide which he named "sinistrin."

No further work on the mucoproteins of these animals was recorded until very recently. Meanwhile the theory of the animal gum seemed to have been entirely discredited, on the one hand, by the failure of Folin in Hammarsten's laboratory to prepare nitrogen-free polysaccharides from several mucoproteins and, on the other hand, by the successful isolation of mucoitin and chondroitin sulfuric acids from many mucoproteins which was accomplished in the laboratories of Hofmeister and of the present writer, in cooperation with López-Suárez particularly.

In the year 1920, for the first time after the date of Hammarsten's publication, reference was made to the carbohydrate complex obtainable from the proteins of *Helix pomatia*. Schmiedeberg³ in a very comprehensive publication on mucoproteins made a statement that from the proteins of *Helix pomatia* a nitrogen-free polysaccharide is obtainable which has the structure of a glucosan-pentosan.

Thus it is seen from this brief review that the work on the mucoproteins of *Helix pomatia* had a great influence on our conception of the structure of mucoproteins in general. Hence, it seemed expedient to test the old conclusions by more modern methods.

Every new work on this problem, of course, has the benefit of the experience of Hammarsten which led to a differentiation

³ Schmiedeberg, O., *Arch. exp. Path. u. Pharmacol.*, 1920, lxxvii, 44.

between the mucoproteins derived from the mucus of these animals and the substances obtained from the foot, on the one hand, and that obtained from the protein glands, on the other. It is unfortunate that the task of dissecting the glands of the animals is too difficult to permit its being carried out on a large scale. The present work was done on a larger scale than that of the older investigators; nevertheless the available material was not sufficiently large to permit as accurate an investigation as is required by modern standards.

Notwithstanding the many shortcomings, the results obtained in the course of the present work are sufficient to warrant several very definite conclusions. First, that the mucoprotein of the mucus belongs to the group derived from mucoitin sulfuric acid. A substance having the properties of the latter was isolated in a state free from protein and perfectly intact in every respect save for a loss of some of its sulfuric acid radicle. It gave on partial hydrolysis a disaccharide (mucosin), and after complete hydrolysis, there were obtained chitosamine and a volatile fatty acid in a proportion equivalent to one acetyl group for each atom of nitrogen. On distillation with hydrochloric acid, it gaveurfural. When mucosin was treated in the same way the yield ofurfural was equal to that required by the theory for equal proportions of glucuronic acid and of chitosamine. Whether the "uronic" acid was actually glucuronic, or galacturonic, or some other acid of this type was as yet not ascertained.

The present substance, like all other mucoitin sulfuric acids, did not reduce Fehling's solution directly, but did so after hydrolysis with mineral acids. It gave a positive test for glucuronic acid with naphthoresorcinol.

Very characteristic for the substance is its solubility. Like all other mucoitin sulfuric acids, it is insoluble in all organic solvents including glacial acetic acid. It is insoluble in water and in alkalies, and is soluble in strong mineral acids. In these solutions the substance was dextrorotatory.

On the basis of solubility the present mucoitin sulfuric acid belongs to the subgroup A, of which the substance derived from "funis mucin" is a representative member.

Thus, the general characteristics of the mucoitin sulfuric acid derived from the mucus of the snails are quite clear and they con-

firm the conclusions expressed in previous publications that mucoproteins secreted by mucous membranes are derivatives of mucoitin sulfuric acid. The interesting point attached to the mucoitin sulfuric acid obtained from the snails is its insolubility in water. Previously, substances of this type were obtained only from certain forms of connective tissue structures. Since the mucoitin sulfuric acid derived from the snails was prepared by a much simpler process than those which led to the soluble forms of mucoitin sulfuric acid, it is suggestive that the soluble mucoitin sulfuric acid is a product of the insoluble form. This suggestion is only tentative.

Somewhat more complex is the problem of the chemical structure of the polysaccharides obtained from the tissues of the snails. It was stated above that Hammarsten was the first to have pointed out that the carbohydrate obtained from the mucoprotein of the foot differed from that prepared from the body of the snail; the first having the properties of the carbohydrate radicle characteristic for other mucoproteins, the second being a distinctive substance. Our observations in certain respects are in harmony with those of Hammarsten. The complex carbohydrate prepared by us from the mucoprotein of the foot of the snails resembled, in the main, the analogous substance prepared from the mucus. The substance prepared from the mucoprotein of the foot, however, contained a small proportion of a different polysaccharide, which is more abundant in the bodies of the snails. As was mentioned above, Hammarsten discovered the polysaccharide and referred to it as "sinistrin." We believe that the material prepared by Hammarsten was purer than ours as we did not attempt the extirpation of the gland with anatomical accuracy. The polysaccharide prepared by us was sparingly soluble in water but readily soluble in strong mineral acids and also to some extent, in alkalies. In the majority of cases the solution was too opaque to permit a very dependable reading of its optical rotation. Whenever it was possible to take the rotation it was found to be to the right, and on a few occasions, the substance was inactive. The optical behavior alone was sufficient to indicate that the substance was not homogeneous. Furthermore, the substance contained small quantities of nitrogen and sulfur, which indicated that it contained a small proportion of mucoitin sulfuric acid.

On hydrolysis with dilute mineral acids, the substance gave a maximum yield of about 60 per cent of a monosaccharide, calculated on the basis of glucose. Besides, on distillation there was obtained between 20 and 30 per cent of acetic acid. The most interesting point in connection with the polysaccharide was the nature of the hexose. This was found to be that of galactose. The configuration of the hexose was suggested first by the phenylsazone prepared from it. This consisted of small platelets, was dextrorotatory, showing, with time, a decline in the numerical value of the rotation. This property is peculiar among hexoses only to phenylgalactosazone and to phenylgulosazone. Besides, the melting point of the osazone was $195^{\circ}\text{C}.$, (uncorrected) which is characteristic for galactosazone. On oxidation with nitric acid, mucic acid was formed, which was identified by its melting point (m.p. $215^{\circ}\text{C}.$), by the absence of optical activity, and by its elementary composition.

Thus, the substance seems to be a polysaccharide composed of galactose, or perhaps of acetyl galactose. It is the first of this kind observed in animal tissues. Galactosans, to the knowledge of the author, have been prepared only from plants in the form of gums. Thus the term animal gum may be well applied to this substance.

The question then arises whether this polysaccharide is actually a component of a "glucoprotein," as Hammarsten suggested, or is a substance which occurs in the tissues of the snails in a free state and is adsorbed to the protein during the precipitation. The latter possibility seems to be suggested by the analytical data on the so called "glucoprotein." Hammarsten and before him, Liechwald and others have found that this protein contained only 1.5 per cent of nitrogen. No other mucoprotein is known with a nitrogen content as low as the one just mentioned. It was therefore attempted to extract the polysaccharide directly from the organs by means of hot water. The method of Pflüger and Jerking was used for separating the polysaccharide from the protein material. Under these conditions, the same polysaccharide was obtained as that from the so called "glucoprotein" of Hammarsten. It was hydrolyzed into galactose and a volatile acid (acetic). This observation was in a way surprising, as Hammarsten reported the isolation of glycogen from the tissues

of the snail. In this respect the experience of Hammarsten was at variance with that of Landwehr. Since the identification and the isolation of glycogen is a very simple matter, it is possible that the presence or absence of glycogen is conditioned by the nutritional state of the animals. It was fortunate for us that in the tissues of our animals, glycogen was completely absent, and this fact facilitated the isolation of the galactose polysaccharide. It is possible, however, that still another polysaccharide is present in the tissues. This possibility is suggested because frequently the osazone prepared from the products of hydrolysis was optically inactive and melted at 202°C. On the other hand, only mucic acid could, with certainty, be isolated from the products of nitric acid oxidation of the polysaccharide. It is unfortunate that the material was not accessible in quantities to permit a more thorough purification of the polysaccharide and of its products of hydrolysis.

EXPERIMENTAL.

PART I.

The work on *Helix aspersa* Müller var. *maxima* Taylor will be reported first in view of the fact that the material was available in larger quantities and therefore permitted more detailed analysis.

Mucoprotein from the Mucus.—This material was collected according to the suggestion of Hammarsten. The shell of the animal was opened and by rubbing the animal with a glass rod it was caused to secrete the mucus. The fresh secretion was transferred into 95 per cent alcohol and kept until a quantity sufficient for work (about 500 gm. of the moist mucoprotein) was collected. In order to facilitate the precipitation of the mucoprotein, the alcohol was acidulated with acetic acid. The mucoprotein was then separated from the alcohol in part by passing through cheese-cloth, in part by filtration. The precipitate was transferred into a fresh portion of alcohol which was refluxed for 2 to 3 hours. The mucoprotein was then freed from the alcohol at first by filtration and subsequently by passing through a hydraulic press. The fairly dry cake was minced in a meat chopper. This material contained a considerable quantity of lime salts and, in order to remove the latter, it was suspended

in large quantities of hot water containing 1 or 2 per cent of acetic acid and the mixture was turbinated. The water was renewed every 2 hours and the extraction continued until the suspended material on incineration left only a very small mineral residue. The suspended material was finally washed with pure water, again filtered, and passed through a hydraulic press.

Mucoitin Sulfuric Acid.

The mucoitin sulfuric acids can be prepared from this material in several ways. Each has its own advantages and the choice of the method should be determined by the purpose for which the material is needed.

First Process.—By this process, the mucoitin sulfuric acid is obtained in the most unaltered state. About 500 gm. of the mucoprotein, still moist, are taken up in 1000 cc. of a 5 per cent solution of sodium hydroxide and the mixture is placed in a shaking machine. After a short interval the mucoprotein is dissolved into a homogeneous viscous fluid. The shaking is continued for 48 hours. At the end of that time the viscous fluid is transferred into twice its volume of 95 per cent alcohol. A precipitate is then formed which is removed by centrifugalization. It is washed repeatedly with 95 per cent alcohol. The precipitate is then suspended in 800 cc. of 5 per cent aqueous sodium hydroxide and again shaken for 24 hours. At this phase the sodium salt of the mucoitin sulfuric acid appears in the main in the form of a suspension. At the end of the 24 hours the mixture is transferred into twice its volume of 95 per cent alcohol. The precipitate is separated from the fluid by centrifugalization. This material is practically insoluble in water. It has a gelatinous character and still gives a positive biuret test, which undoubtedly is due to the soluble protein which adheres mechanically to the mucoitin sulfuric acid. In order to remove the traces of the adhering soluble protein, the material is suspended in 5 per cent aqueous sodium hydroxide and placed in the shaking machine for an hour. After that time the mucoitin sulfuric acid is sedimented by centrifugalization. The alkali is replaced by distilled water and the suspension again is placed in a shaking machine. The latter operation is repeated as long as the mucoitin sulfuric acid gives a positive biuret test. The final product is biuret-

free but still contains some calcium. In order to remove the latter, hydrochloric acid is added very cautiously to a suspension of the mucoitin sulfuric acid in water until the mixture reacts acid to Congo red. The suspension is then placed in a shaking machine for about $\frac{1}{2}$ hour. The dilute hydrochloric acid is replaced by distilled water and the shaking with fresh portions of distilled water is repeated until the washings no longer contain chlorine ions. The final product is dried by treatment with alcohol and ether. The average yield from 500 gm. of moist mucoprotein is from 15 to 18 gm. of mucoitin sulfuric acid.

Second Process.—This process is much less laborious and time-consuming but has the disadvantage that it leads to a smaller yield and to a product which contains less sulfur than the substance obtainable by the first process.

The mucoprotein is taken up in twice its weight of 5 per cent solution of sodium hydroxide and placed on a water bath for 3 hours. The clear solution is then cooled and neutralized with acetic acid. A precipitate consisting chiefly of protein material is formed and removed by centrifugalization. From the supernatant liquid the mucoitin sulfuric acid is precipitated by means of basic lead acetate and ammonia. This precipitate is freed from supernatant liquid by centrifugalization. The precipitate is washed several times with water and is then taken up in enough concentrated hydrochloric acid to convert all the lead into its chloride. The lead chloride is removed by centrifugalization and the supernatant liquid is poured into a large excess of glacial acetic acid. The mucoitin sulfuric acid settles out in the form of a flocculent precipitate. This again is freed from the supernatant liquid by centrifugalization. It is taken up in a little water and precipitated by means of alcohol. By this process protein-free mucoitin sulfuric acid can be prepared in about 48 hours. The maximum yield obtainable by this process was 10 gm. out of 500 gm. of moist mucoprotein.

A substance which as regards its sulfur content occupies a position intermediate between the other two is obtained when the lead salt is suspended in water containing barium acetate and a stream of hydrogen sulfide gas is passed through the mixture. The filtrate is then concentrated to a very small volume and is poured into a large excess of glacial acetic acid. The product

obtained in this way is taken up in water and reprecipitated with alcohol.

Properties of the Mucoitin Sulfuric Acid.—As was already stated by each one of the above methods, a biuret-free substance is obtainable. It is very little soluble in water, in aqueous alkalis, or in dilute mineral acids. It is soluble in concentrated mineral acids giving a slightly opalescent solution. The substance does not reduce Fehling's solution but shows a strong reduction after preliminary hydrolysis. The substance gives with naphthoresorcinol a very strong test for glucuronic acid. The best samples prepared by the first process contained only half the theoretical amount of sulfuric acid; on the other hand, after refluxing for 1 hour over a free flame with a solution of 20 per cent of oxalic acid, a product is obtained which still contains 1 per cent of sulfur.

Composition of the Various Samples of Mucoitin Sulfuric Acid.

Sample I prepared by the first process:

0.1000 gm. substance required for neutralization (Kjeldahl) 1.90 cc. 0.1 N acid. N 2.66.

0.2048 gm. substance: 0.0350 gm. BaSO_4 . S 2.34.

Sample II prepared by the second process:

0.0924 gm. substance: 0.1370 gm. CO_2 and 0.0468 gm. H_2O . C 40.43, S 5.66.

0.1626 gm. substance neutralized (Kjeldahl) 3.0 cc. of 0.1 N acid. N 2.58.

0.1635 gm. substance: 0.0136 gm. BaSO_4 . S 1.14.

Sample III prepared according to the third process:

0.1000 gm. substance neutralized (Kjeldahl) 3.50 cc. of 0.1 N acid. N 2.490.

0.2590 gm. substance: 0.0312 gm. BaSO_4 . S 1.65.

$\text{C}_{28}\text{H}_{48}\text{O}_{29}\text{N}_2\text{S}_2$. Calculated. C 35.72, H 5.18, N 2.99, S 6.82.

Found. Sample I. " 2.66, " 2.34.

" II. " 40.43, " 5.66, " 2.58, " 1.14.

" III. " 4.90, " 1.65.

Mucosin.

It was pointed out in previous publications on mucoitin sulfuric acids that mucosin was much less stable than chondrosin, and that great caution must be exercised not to permit the hydrolysis to pass beyond the stage of mucosin formation. To avoid the latter danger, it was attempted to hydrolyze the mucoitin

sulfuric acid by means of oxalic acid. 10 gm. of the substance were taken up in 200 cc. of a 20 per cent solution of oxalic acid and refluxed over a free flame for 1 hour. At the end of that time the heavy floccules of the mucoitin sulfuric acid disappeared leaving only a fine white precipitate consisting, in the main, of calcium oxalate. The solution was practically colorless. It was freed from the sediment by centrifugalization and was poured into 5 volumes of a mixture consisting of equal parts of alcohol and ether.

In this manner a substance was obtained which was more soluble than the original substance. The substance had 50 per cent of its nitrogen in the form of amino nitrogen and still contained 1 per cent of sulfur. The yield of the substance was 5 gm. from 10 gm. of the original material. These 5 gm. were then dissolved in 100 cc. of 10 per cent hydrochloric acid, and refluxed for $\frac{1}{2}$ hour on a boiling water bath. The solution was then concentrated under reduced pressure to 5 cc. and poured into 600 cc. of a mixture of equal parts of alcohol and ether. The material obtained after this operation differed only little from the original. The proportion of amino to total nitrogen had increased to about 60 per cent. The yield from 5 gm. was 4.8 gm. This material was then dissolved in 50 cc. of 20 per cent hydrochloric acid and treated as before; 1.2 gm. of mucosin were obtained. The substance was slightly colored and could not be used for optical measurement. 0.050 gm. of the substance had the reducing power of 0.027 gm. of glucose.

The substance contained 4.69 per cent of total nitrogen and 4.02 per cent of amino nitrogen. 0.400 gm. of the substance gave on distillation 0.065 gm. of phloroglucide of furfural, which corresponds to 50 per cent of glucuronic acid.

Thus the substance had the properties of mucosin but was as yet crude. Unfortunately, further purification was impossible because of the lack of material.

Chitosamine.

5 gm. were taken up in 25 cc. of 20 per cent hydrochloric acid to which 2 gm. of barium chloride and 1 gm. of stannous chloride had been added and refluxed over a free flame. The solution was then freed from barium and from tin and concentrated under

reduced pressure. Chitosamine hydrochloride crystallized out. The substance turned dark above 200°C., but had no melting point. It analyzed as follows:

No. 144. 0.0100 gm. substance: 1.20 cc. nitrogen gas (Van Slyke).

$C_6H_{13}O_6N \cdot HCl$. Calculated. N 6.51.

Found. " 6.73.

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{\text{Initial. } +0.98^\circ \times 100}{1 \times 1} = +98^\circ \quad [\alpha]_D^{20} = \frac{\text{Equilibrium. } +0.76^\circ \times 100}{1 \times 1} = +76^\circ$$

Furfural.

The distillation of furfural was conducted under the usual conditions. The yield of the phloroglucide was much below that required by theory. One of the reasons, however, might have been the fact that only small quantities of material were available for these experiments. On the other hand, from mucosin, the yield of the phloroglucide of furfural agreed with that required by the theory. Thus, 0.5000 gm. of the crude product yielded 0.035 gm. of phloroglucide of furfural, which corresponds to 0.100 gm. or 20 per cent of glucuronic acid, whereas the theory requires 40 per cent.

Estimation of the Volatile Fatty Acids.

The volatile fatty acids were estimated in two ways. In one case, the material was suspended in an excess of barium hydroxide and refluxed over a free flame in an apparatus protected against the absorption of carbon dioxide from the air. The operation was continued for 4 hours at the end of which time it was interrupted, and the material allowed to cool. The mixture was then made acid to Congo red by means of sulfuric acid and distilled. The distillate was received in 0.1 N alkali protected against the absorption of carbon dioxide from the air.

Either 1 or 0.500 gm. of the material was used for distillation. Calculated for acetic acid, the yields varied between 10 and 15 per cent. The yield required by theory for free chondroitin sulfuric acid is 12.6 per cent. The small amount of available

material did not permit the isolation and the identification of the acid.

Polysaccharide from the Mucoproteins of the Foot of the Snails.

From the foot of the snails the mucoprotein was extracted according to the directions of Hammarsten. The minced organs were extracted with a 0.1 per cent solution of sodium hydroxide, the solution centrifugalized, and the mucoprotein precipitated from the supernatant liquid by means of acetic acid. The precipitate thus formed was redissolved and reprecipitated several times. The final product contained 14 per cent of nitrogen. The final precipitate of the mucoprotein was refluxed with 95 per cent alcohol for several hours then taken up in alkali, and further treated exactly according to the second process described above and the product obtained from it was undoubtedly a mixture of mucoitin sulfuric acid with the polysaccharide. The proportion of the former was undoubtedly very large. This is quite natural since the dissecting of the foot from the body was not carried out with anatomical accuracy. The composition of one of the samples obtained by this process was the following.

C 40.39, H 6.19, N 3.58, S 0.48.

The substance gave a negative biuret test and with naphthoresorcinol a strong test for glucuronic acid.

Polysaccharide from the So Called "Glucoprotein."

The so called "glucoprotein" was prepared from the bodies of the snails in exactly the same manner as the mucoprotein from the foot. It contained 12 per cent of nitrogen. The carbohydrate was isolated by the second process employed for the preparation of mucoitin sulfuric acid from the mucoprotein of the mucus. The final product obtained in this manner was a slightly grayish white powder. It was never completely soluble in water. The solution was practically always opaque. The substance was completely soluble in hydrochloric acid of specific gravity 1.19 and from this solution it is precipitated by means of alcohol. In hydrochloric acid solution the substance is slightly dextrorotatory. Some samples appeared entirely inactive. The sub-

stance always contained small proportions of nitrogen and sulfur, though the biuret test was apparently negative. No perceptible test for glucuronic acid could be obtained with naphthoresorcinol; also with orcinol the test was negative. The yield of furfural on distillation was negligible. The small proportions of nitrogen and sulfur (when present) were undoubtedly due to insignificant admixtures of mucoitin sulfuric acid. The substance gave with iodine a negative test for either starch or dextrin.

Samples were obtained containing as little as 0.3 to 0.6 per cent of nitrogen and contained no sulfur.

The sample which was used for the experiments described below had the following composition. The purer samples were too small for further work.

Sample No. 598. C 42.61, H 6.38, N 0.98, S 0.67.

Hydrolysis of the Polysaccharide.

1 gm. of the substance was hydrolyzed by refluxing over a free flame with 100 cc. of 4 per cent sulfuric acid. The resulting perfectly colorless solution was dextrorotatory ($[\alpha] = +0.32^\circ$) and reduced Fehling's solution equivalent to 0.586 gm. of glucose.

From this solution an osazone was obtained which crystallized from pyridine and alcohol. It crystallized in bright platelets melting at 197° (uncorrected).

The rotation of the osazone in pyridine and alcohol solution ($c = 1$ per cent; $l = 50$ mm.).

$$\begin{array}{c} \text{Initial.} \\ \alpha_D^{20} = +0.35^\circ \end{array}$$

$$\begin{array}{c} \text{Equilibrium.} \\ \alpha_D^{20} = +0.16^\circ \end{array}$$

The rotation and the direction of the mutarotation of the substance suggested that it was a phenylgalactosazone.

From samples which contained more impurities the osazone was inactive and in such cases, the melting point was somewhat higher; namely, 202°C .

Nitric Acid Oxidation.

3 gm. of the polysaccharide were hydrolyzed by refluxing for 8 hours over a free flame in 150 cc. of 10 per cent nitric acid. The solution was then concentrated to 30 cc., an equal volume of

nitric acid of specific gravity 1.4 was added, and the solution was allowed to stand overnight and then rapidly oxidized. From this material 0.500 gm. of mucic acid was obtained. The substance was optically inactive, melted at 215°C., and had the following composition.

0.1000 gm. substance: 0.1252 gm. CO₂ and 0.0442 gm. H₂O.

C₆H₁₀O₅. Calculated. C 34.22, H 4.80.

Found. " 34.14, " 4.95.

Acetyl Estimation.

1 gm. of the material was used for acetyl estimation by the barium hydroxide hydrolysis and subsequent distillation of the hydrolysate acidulated with sulfuric acid as described in an earlier section of this paper. The yield was 0.200 gm. of acetic acid which was identified as the silver salt. For analysis it was recrystallized from water.

0.050 gm. substance: 0.0324 gm. Ag.

C₂H₃O₂Ag. Calculated. Ag 64.31.

Found. " 64.80.

Polysaccharide Obtained Directly from the Bodies of the Snails.

The initial stages of the isolation of this polysaccharide were those recommended by Pflüger and Nerking for the isolation of glycogen. The minced material was allowed to stand on the boiling water bath for 3 hours with 2 volumes of hot water. The mixture was then made to contain 3 per cent of potassium hydroxide and 8 per cent of potassium iodide and to the mixture alcohol was added as long as it continued to produce precipitation. The precipitate was collected by centrifugalization and was washed several times with a 50 per cent solution of alcohol containing 3 per cent of alkali and 8 per cent of potassium iodide.

Further purification was very tedious. The crude material was dissolved in hydrochloric acid of specific gravity 1.19, care being taken to avoid warming the solution. The solution (cooled in an ice and salt mixture) was centrifugalized and the supernatant liquid was poured into alcohol. The precipitate was then centrifugalized and washed with alcohol until all the free mineral acid was removed. The precipitate was then suspended

n water and reprecipitated by alcohol. In this manner the still adhering traces of hydrochloric acid were removed completely. The product obtained in this manner was a mixture of the new polysaccharide and mucoitin sulfuric acid.

For the separation of the two, advantage is taken of the differences in their solubilities. The mucoitin sulfuric acid is the more insoluble product, and the polysaccharide is more soluble. The product just described was taken up in water and centrifugalized. From the supernatant liquid, the polysaccharide was precipitated by means of alcohol. The insoluble fractions could be purified by taking up in a little hydrochloric acid so that only part went into solution. The insoluble part contained 3.15 per cent of nitrogen and 1.5 per cent of sulfur.

The polysaccharide was again dissolved in water and centrifugalized, and the supernatant liquid was precipitated with alcohol.

The final product had the same properties as the polysaccharide obtained from the so called "glucoprotein." It also contained a small admixture of mucoitin sulfuric acid which could undoubtedly be entirely removed if sufficient material were available.

Sample I. 0.0925 gm. substance required (Kjeldahl) 0.65 cc. 0.1 N alkali.
 0.2041 gm. substance: 0.0100 gm. BaSO₄ (Carius).
 0.1052 " " : 0.1644 gm. CO₂ and 0.0600 gm. H₂O.
 Found. C 42.61, H 6.38, N 0.98, S 0.67.

Further samples were obtainable when the hot aqueous extracts of the bodies were removed by centrifugalization and only the residues were treated as above. The product obtained in this manner contained practically no sulfur and only minimal proportions of nitrogen, as is illustrated by the following two samples.

No. 572. 0.1000 gm. substance required for neutralization (Kjeldahl) 20 cc. 0.1 N alkali. N 0.28.

No. 580. 0.1000 gm. substance required for neutralization (Kjeldahl) 30 cc. 0.1 N alkali. N 0.42.

1 gm. of the substance containing 0.9 per cent of nitrogen was hydrolyzed with 100 cc. of a 4 per cent solution of sulfuric acid. The solution was dextrorotatory ($\alpha = +0.60^\circ$ in a 100 mm. tube) and contained 0.6287 gm. of reducing sugar, calculated on the basis of glucose. (The volume of the solution was 87 cc.)

The osazone prepared from it was optically inactive and had a melting point of 202°C.

No. 614. 0.0626 gm. substance: 8.70 cc. of nitrogen gas at 28°C. and 756.2 mm.

$C_{18}H_{22}O_4N_4$.	Calculated.	N 15.63.
	Found.	" 15.69.

Oxidation with Nitric Acid.

3 gm. of the substance were hydrolyzed by refluxing over a free flame for 8 hours with 10 per cent nitric acid and the operation was repeated as in the above described experiment. The yield of crude mucic acid was 0.565 gm. For analysis it was twice recrystallized out of water. It was optically inactive, had a melting point of 215°C., and had the following composition.

0.1040 gm. substance: 0.1298 gm. CO_2 and 0.0458 gm. H_2O .

$C_6H_{10}O_8$.	Calculated.	C 34.22, H 4.80.
	Found.	" 34.03, " 4.91.

It is possible that the mother liquor from mucic acid contained saccharic acid. The mother liquor from mucic acid was rendered alkaline by means of potassium hydroxide and the solution was then acidulated with acetic acid. On addition of a little alcohol and subsequent scratching the walls of the beaker with a glass rod a crystalline deposit began to form.

The acid potassium salt was then converted into the silver salt. Two fractions were obtained which were analyzed without recrystallization. One (the major portion) contained 54.0 per cent of silver, the other, 62.0 per cent of silver. The silver salts of saccharic and anhydrosaccharic acids contain 50.95 and 53.2 per cent of silver respectively. Silver oxalate contains 71.0 per cent. Since the mother liquor from the mucic acid did contain some oxalic acid, it is not possible for the present to interpret the significance of these silver salts. If saccharic acid were present, it may be derived from chitosamine, since the polysaccharide still contained a small impurity of mucoitin sulfuric acid.

PART II.

Helix pomatia.

This snail was available in smaller quantities. However, practically all that was observed on the *Helix aspersa* was noted also on *Helix pomatia*. The mucoprotein from the mucus contained a mucoitin sulfuric acid, the so called "glucoprotein." However, in this case, no attempt was made to isolate the polysaccharide directly from the bodies of the snails.

The procedures for isolation and analysis were exactly the same as described in the first part of the paper and will therefore be omitted in this place. Only the analytical results will be reported.

Mucoitin Sulfuric Acid Obtained from Mucoprotein of the Mucins.

The substance was obtained by the two procedures described in the first part.

One of the samples obtained by the first process was converted into barium salt and analyzed as follows:

0.2000 gm. substance neutralized (Kjeldahl) 3.65 cc. 0.1 N acid. N 2.32.

0.2086 gm. substance: 0.0762 gm. BaSO₄. S 5.37.

0.2054 " " : 0.0294 " " Ba 9.01.

C₂₃H₄₄O₂₉N₂S₂Ba₂. Calculated. N 2.32, S 5.30, Ba 22.70.

Found. " 2.32, " 5.37, " 9.01.

The composition of the products varied from one experiment to the other.

Chitosamine.

The chitosamine obtained from this material had the following composition.

0.020 gm. substance: (Van Slyke) 2.35 cc. nitrogen gas at 25°C. and 769.9 mm.

C₆H₁₃O₅N·HCl. Calculated. N 6.51.

Found. " 6.61.

The optical rotation of the substance in 2.5 per cent hydrochloric acid was

$$[\alpha]_D^{25} = \frac{\text{Initial. } +0.96^\circ \times 100}{1 \times 1} = +96^\circ \quad [\alpha]_D^{25} = \frac{\text{Equilibrium. } +0.76^\circ \times 100}{1 \times 1} = +76^\circ$$

The mucoitin sulfuric acid gave on distillation 8 per cent of the phloroglucide of furfural, which corresponds to about 24 per cent of glucuronic acid. The acetyl value corresponded to 15 per cent. The acetic acid was not isolated.

Polysaccharide from the So Called "Glucoprotein."

The polysaccharide was optically inactive and had the following composition. The purest samples contained 0.3 and 0.4 per cent of nitrogen. The larger sample had the following composition.

C 36.88, H 5.09, N 1.2, S 0.8, and 8 per cent ash.

1 gm. on hydrolysis gave in 84 cc. a dextrorotation of $\alpha_D = +0.46^\circ$ and reducing power equivalent to 0.560 gm. of glucose. The phenylosazone prepared from it had the appearance of a galactose. It had in pyridine-alcohol solution ($c = 1$ per cent, $l = 50$ mm.), an initial rotation of $\alpha_D = +0.10^\circ$ and an equilibrium rotation of $\alpha_D = +0.10^\circ$.

0.0748 gm. substance: 10.20 cc. nitrogen gas at 26.0°C . and 7.53 mm.

$\text{C}_{18}\text{H}_{22}\text{O}_4\text{N}_4$. Calculated. N 15.63.

Found. " 15.45.

1 gm. of the substance was oxidized with nitric acid and gave about 0.200 gm. of mucic acid. The substance was optically inactive and had a melting point of 215°C .

CONCLUSIONS.

1. The carbohydrate radicle isolated from the mucoproteins of the mucus of *Helix aspersa* and *Helix pomatia* belongs to the group of mucoitin sulfuric acid. On partial hydrolysis the polysaccharides yield the disaccharide mucosin. From the product of complete hydrolysis there were isolated sulfuric acid, chitosamine, and a volatile fatty acid. On distillation with hydrochloric acid, the mucosin yielded furfural in a quantity required by the theory of the structure of this disaccharide.

2. From the bodies of the snails a substance is obtainable which may be regarded as animal gum and which consists largely of a polygalactose, perhaps of an acetylated polygalactose.

3. From the bodies of the snails by mere extraction with water and by further treatment by the process of Pflüger and Nerking, an identical polysaccharide can be isolated.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

VIII. THE DISTRIBUTION OF HYDROGEN, CHLORIDE, AND BICARBONATE IONS IN OXYGENATED AND REDUCED BLOOD.

By DONALD D. VAN SLYKE, A. BAIRD HASTINGS,
CECIL D. MURRAY, AND JULIUS SENDROY, JR.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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The purpose of this paper is to present data showing the effects of changes in blood reaction and oxygenation on the distribution ratios of hydrion activities and bicarbonate and chloride concentrations between serum and cells, and to discuss the relative activity coefficients of the Cl and HCO_3 anions in serum and cells.

In the fifth paper of this series (1) the laws governing the electrolyte and water distribution between cells and serum in the blood were formulated, and subjected to experimental verification. It was shown that as a first approximation

$$r = \frac{[\text{H}']_s}{[\text{H}']_c} = \frac{[\text{Cl}]_c}{[\text{Cl}]_s} = \frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s} = 1 - f[\text{BHb}]_c$$

where $[\text{BHb}]_c$ is the amount of base bound by hemoglobin in the cells. The value of $f[\text{BHb}]_c$ was expressed in their Equations 10 and 14 of that paper. In the development of these equations it was pointed out that the assumption of complete dissociation of the electrolytes served only for a first approximation. However, if the dissociation (or activity) of the electrolytes in cells and serum were nearly equal it could be expected that the equation would hold approximately. This was found to be the case for the distribution of Cl and HCO_3 in oxygenated blood under varying CO_2 tensions, and for the distribution of H' as calculated from the CO_2 contents by Hasselbalch's equation, $\text{pH} = \text{pK}_1' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$.

In the present paper we have determined the distributions not only in oxygenated but also in reduced blood, in order to ascertain whether the predicted effect of reduction (causing r to move nearer to 1 as a result of the lesser amount of base bound by the hemoglobin) occurs. As will be shown in the experimental part, it does occur, and approximates the magnitude calculated from the effect of oxygenation and reduction on the base bound by the hemoglobin.

We have also determined the H^+ activities in reduced cells and serum directly by the electrometric method, and determined the pK' of Hasselbalch's equation for cell contents. By using this pK' in calculating the H^+ activities by Hasselbalch's equation in oxygenated blood, where the hydrogen electrode cannot be used, the activities thus estimated could be placed on a basis of measured electrometric H^+ activity values. From the relationship between the distribution ratios of H^+ activities and Cl and HCO_3 concentrations between serum and cells we have attempted to estimate the relative activity coefficients for Cl' and HCO_3' in serum and cells. The importance of such estimates was recognized by Warburg (2). We have attempted also to determine the Cl' activities directly by the electrometric method. The difficulties of doing so in the presence of the blood proteins, which Warburg (2) has discussed, have thus far prevented success at this point.

For the determination of chlorides in the cells Van Slyke, Wu, and McLean (1) did not possess a method of satisfactory accuracy. They therefore estimated the initial cell Cl content by difference from whole blood and serum analyses, and calculated the subsequent cell chloride variations, caused by varying CO_2 tensions, from the variations in serum Cl, for which a highly accurate method was available. Accordingly, as they pointed out, their chloride analyses afforded accurate data for the *variation* of the Cl ratio with changing pH, but could be accepted only as approximate for the absolute values of the $[\text{Cl}]_c:[\text{Cl}]_s$ ratio. The accurate nitric acid ashing method for cell chlorides (3) developed in the meantime has been used in the present experiments, and we believe the absolute values of the chloride ratios, as well as the shifts in them, given in this paper are fairly reliable. They

indicate in agreement with Warburg (2) a $[\text{Cl}]_c:[\text{Cl}]_s$ ratio curve definitely lower than the $[\text{HCO}_3]_c:[\text{HCO}_3]_s$ curve.

Symbols and Equations.

The symbols used in the present paper are the following.

r = Donnan distribution ratio of diffusible ions between serum and cells.

γ = activity coefficient of ions.

α = activity of ions.

C = molal concentration.

V = valence.

μ = ionic strength.

$[\text{Cl}]$ = Cl molality in millimols of chloride per kilo of water.

$[\text{HCO}_3]$ = HCO_3 molality in millimols of bicarbonate per kilo of water.

s = as a subscript refers to serum.

c = as " " " " cells.

$(B)_s$ = milli-equivalents of serum base per kilo of whole blood.

$(B)_c$ = " " " " " " " " " "

$(BP)_s$ = " " " base bound to serum protein per kilo of whole blood.

$(BP)_c$ = milli-equivalents of base bound to cell protein per kilo of whole blood.

(Hb) = millimols of hemoglobin per kilo of whole blood.

$(\text{H}_2\text{O})_s$ = kilos of serum water " " " " "

$(\text{H}_2\text{O})_c$ = " " cell " " " " " "

P_{CO_2} = tension of CO_2 in millimeters of mercury.

$\text{pH}^* = \log \alpha_{\text{H}^+}$.

$\text{pX} = -\log X$ in general.

*Sørensen and Linderström-Lang (4) have recently reviewed the various standards upon which pH measurements have been based and have made the following recommendations regarding nomenclature and consistency in calculation. When the 0.1 N calomel electrode and the Bjerrum extrapolation is used the value of the calomel electrode at 18° is taken to be 0.3380 volts, if hydrogen ion values are to be consistent with the hydrogen ion concentration of hydrochloric acid solutions as determined from conductivity measurements. This hydrogen ion value in the logarithm form is to be designated by pH as in the past. If on the other hand, hydrogen ion values are to be consistent with activity values for hydrochloric acid, then the value of the calomel electrode is taken to be 0.3357 at 18°. When this is done Sørensen and Linderström-Lang recommend that the logarithm of the hydrogen ion value be designated by $\text{p}\alpha_{\text{H}}$. In their proposed nomenclature $\text{p}\alpha_{\text{H}} = \text{pH} + 0.04$.

In the previous papers of this series we have based our pH values on

The following basic equations are used, and will be referred to in the text.

$$(1) \quad r = 1 - \frac{(\text{BP})_c + (\text{Hb})}{2 (\text{B})_c - (\text{BP})_c + (\text{Hb})} + \frac{(\text{BP})_s}{2 \{ (\text{B})_s - (\text{BP})_s \}}^1$$

$$\gamma = \frac{\alpha}{C}$$

$$\mu = \frac{1}{2} \Sigma CV^2$$

$$(2) \quad \log \gamma = -\beta V^2 \sqrt{\mu}$$

$$(3) \quad \frac{[\alpha_{\text{H}}] \times [\alpha_{\text{HCO}_3}']}{[\alpha_{\text{H}_2\text{CO}_3}]} = K_1$$

$$\text{pH} + \text{p}\alpha_{\text{HCO}_3}' - \text{p}\alpha_{\text{H}_2\text{CO}_3} = \text{p}K_1$$

activity values of hydrochloric acid, so that our pH values have been equal to Sørensen and Linderström-Lang's $\text{p}\alpha_{\text{H}}$ values. In order to avoid the confusion of change in nomenclature in this series of papers, we shall continue to use the symbol pH to express the negative logarithm of H^+ activity.

¹ Equation 1 is Equation 14 of Van Slyke, Wu, and McLean. In their derivation of it one factor was omitted without the explanation which should be given. The value of $\frac{(\text{H}_2\text{O})_s}{(\text{H}_2\text{O})_c}$ was shown to equal $\frac{2(\text{B})_s - (\text{BP})_s}{2(\text{B})_c - (\text{BP})_c + (\text{Hb})}$ and the latter was then substituted for $\frac{(\text{H}_2\text{O})_s}{(\text{H}_2\text{O})_c}$ in their Equation 12. The resulting equation is $r = 1 - \frac{2(\text{B})_s - (\text{BP})_s}{2(\text{B})_s - 2(\text{BP})_s} \times \frac{(\text{BP})_c + (\text{Hb})}{2(\text{B})_c - (\text{BP})_c + (\text{Hb})} + \frac{(\text{BP})_s}{2(\text{B})_s - (\text{BP})_s}$. In their Equation 14 the factor $\frac{2(\text{B})_s - (\text{BP})_s}{2(\text{B})_s - 2(\text{BP})_s}$ is omitted, as though the numerator and denominator exactly cancelled, which is, of course, impossible. However, the value of $(\text{BP})_s$ is only about 0.04 of $2(\text{B})_s$ so that the effect of the factor $\frac{2(\text{B})_s - (\text{BP})_s}{2(\text{B})_s - 2(\text{BP})_s}$ is only to decrease the term subtracted from 1 by about 4 per cent, and to increase the value of r by from 1 to 2 per cent. For the purposes of the paper such a factor could be neglected, and the equation, simplified by omitting it, was given as above.

$$(4) \quad \text{pH} + \text{p}[\text{HCO}_3] - \text{p}[\text{H}_2\text{CO}_3] = \text{pK}_1 - \text{p}\gamma_{\text{HCO}_3} = \text{pK}_1'$$

$$(5) \quad \text{p}\gamma_{\text{HCO}_3} = -\log \gamma_{\text{HCO}_3} = \text{pK}_1 - \text{pK}_1'$$

$$(6) \quad [\text{H}_2\text{CO}_3] = \frac{P_{\text{CO}_2} \times 0.555}{760 \times 0.0224} = 0.0326 P_{\text{CO}_2}$$

Ionic Strength of Serum.

Donnan's law expressing the distribution of diffusible ions between two solutions separated by a membrane impermeable to one or more ions was developed from thermodynamic reasoning applied to ideal solutions, in which it is assumed "that the electrolytes are completely ionized and that the ions act like ideal solutes. The exact equations can, however, be stated only in terms of the chemical potentials of Willard Gibbs or of the ion activities or ionic activity coefficients of G. N. Lewis" (5).

Donnan's equations apply therefore to the effective concentrations or activities of the diffusible ions rather than their actual concentrations. (The nature of ionic activity and its relationship to ionic concentration are discussed in Lewis and Randall's "Thermodynamics" (6), particularly in Chapters XXII, XXVI, and XXVIII.) The conception of ionic activity presented by Lewis, and apparently concordant with as many of the known facts as any present conception, is that all strong electrolytes are completely dissociated in solution, but that forces acting between the ions prevent them from exhibiting the same activities that they have at infinite dilution. Lewis speaks of the activity as the fugacity or escaping tendency of a solute as compared with the fugacity in the standard state. (In the case of a solute gas the fugacity is exemplified by its tension.) For solutes in solution the standard state is considered to be that of a solution such that $\frac{\alpha}{m}$ at infinite dilution, where α is the activity and m the molality.

Perhaps a more tangible conception than the above sketched from Lewis and Randall is that the activity of the ions in a given solution may be expressed as the ratio between the amount of work the ions will do on dilution to infinite volume and the amount of work that the same number of molecules of an ideal gas would do in expanding similarly from the same initial volume.

The interionic forces opposing removal of the ions from each other diminish the work the latter do on dilution, diminish their fugacity, and evince themselves by their effects on such solution properties as osmotic pressure and electrical conductivity.

Milner from mathematical considerations and Debye and Hückel from kinetic premises arrived at nearly the same expression for the change in the free energy of a dilute solution of a strong electrolyte when it is infinitely diluted (A. A. Noyes (7)). Taking into account only the effect of the inter-ionic forces, this expression leads to the following relationship between the activity coefficient of any ion (activation, Noyes (7)) and the total ionic strength of the solution.

$$(2) \quad \log \gamma = -\beta V^2 \sqrt{\mu} \text{ when } \mu < 0.10$$

Some uncertainty exists regarding the exact value of β , Debye and Hückel's derivation leads to the value 0.50 which coincides with the figure obtained by Brönsted and La Mer (8) from solubility measurements; Milner's derivation leads to a value of about 0.34, and A. A. Noyes thinks that the most probable value is approximately 0.42. As a matter of fact the value actually found experimentally is not exactly identical for all ions, though of the same order of magnitude: *e.g.* β for Cl is approximately 0.33 (calculated from Lewis and Randall²) whereas for HCO_3 it is 0.50 (Hastings and Sendroy (9)).

A more complete expression valid over a wider range of ionic concentrations has been recently given by Hückel (10).

$$\log \gamma = -\frac{\beta \sqrt{\mu}}{1 + A \sqrt{\mu}} + C \mu$$

where A is a function of the ionic diameters and C is a function of the dielectric properties of the solution. Since no information is as yet available concerning the factors A and C in protein solutions it is impossible to utilize the equation at the present time in our system. A first approximation of γ from the ionic strength of the serum and cells has been attempted, however, by the use of the simplified Equation (2) $\log \gamma = -\beta V^2 \sqrt{\mu}$.

To calculate the activity coefficient of strong electrolytes in

² Lewis and Randall (6), p. 382.

serum from Equation 2 it is necessary first to obtain an approximate value for the ionic strength of the serum. This has been done for horse serum at pH 7.40 from typical analyses of the constituents and leads to the value $\mu_s = 0.167$. These data, given in Table I, are self-explanatory except in the case of the rôle attributed to the proteins. We have assumed a protein concentration of 80 gm. per kg. of water. The amount of base bound at pH 7.4 is 0.18 millimol per gm. of serum protein (Van Slyke, Wu, and McLean (1)). This means that 0.0144 equivalent of base per kg. of water is bound to protein. It is impossible at the present time to state exactly the effect of proteins on the ionic strength of the serum for the following reasons: The pro-

TABLE I.

Calculation of Ionic Strength of Normal Horse Serum at pH_s 7.40.

Basic constituents.	Mols Kg. H ₂ O	Acidic constituents.	Mols Kg. H ₂ O
Total.....	0.160	Total.	0.160
Na.....	0.1451	Cl	0.103
K.....	0.0065	HCO ₃	0.029
Ca.....	0.0032	SO ₄	0.002
Mg.....	0.0010	HPO ₄	0.001
		H ₂ PO ₄	0.0002
		Protein.	0.0144
			0.0074

$$\mu_s = \frac{1}{2} \Sigma CV^2 = 0.167.$$

tein molecule is a large one, and the charges due to ionization of the constituent groups are probably so far removed from one another that they cannot be considered to affect the activity of ions in the same manner as other polyvalent ions. It is possible that the surface density of the charges is more significant than the charges per molecule. In the absence of more exact information on this point we have assumed in this calculation that the proteins act as if univalent. Further evidence that the ionic strength of serum is approximately 0.167 is given by the fact that the experimentally determined pK_1' of carbonic acid in serum is 6.13 and that this corresponds to the calculated pK_1' of carbonic acid in a salt solution of ionic strength 0.160.

Using the value $\mu_s = 0.167$ and the equation $-\log \gamma_s = 0.5 V^2 \sqrt{\mu} = 0.5 \sqrt{\mu}$ one may calculate the activity coefficient of HCO_3 ions in serum to be $\gamma_s = 0.625$. As will be seen, this agrees well with the γ value estimated as $\gamma = \frac{K_1}{K_1'}$, where K_1 is the dissociation constant of carbonic acid, and $K_1' = \alpha_{\text{H}} \cdot \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$

Ionic Strength of Cells.

The calculation of the ionic strength of cell fluid presents greater difficulty. In Table II we have presented the data used in its

TABLE II.

Calculation of the Ionic Strength of Normal Oxygenated Horse Cells at pH_c 7.15.

Basic constituents.	Mols Kg. H ₂ O	Acidic constituents.	Mols Kg. H ₂ O
Total $\frac{\text{Mols}}{\text{Kg. H}_2\text{O}}$	0.170	Total $\frac{\text{Mols}}{\text{Kg. H}_2\text{O}}$	0.170
Na*		Cl	0.0800
K	0.170	HCO_3	0.0230
Ca*		SO_4	0.0008
Mg*		Hb	0.0500
		X(HPO_4 , H_2PO_4 , etc.)	0.0154

$$\mu_c = \frac{1}{2} \Sigma CV^2 = 0.171$$

* Bunge reports no Na, Ca, or Mg in horse cells.

calculation from analysis. As in the case of the serum proteins we have assumed that hemoglobin functions as a univalent component in contributing to the ionic strength of the cell fluid. Calculated in this manner the ionic strength, $\mu_c = 0.171$. The activity coefficient of monovalent ions estimated from the equation $-\log \gamma_c = 0.5 \sqrt{\mu_c}$ is $\gamma_c = 0.62$, practically the same as the serum.

Several assumptions have been made in these calculations which render the values of γ_s and γ_c obtained merely first approximations. We have assumed first, that we are dealing with solutions of strong electrolytes; second, that the solutions are not

so concentrated that the size of the ions plays a significant rôle; and third, that the dielectric properties of the solutions are not different from those of water. In the case of serum the results make it probable that these assumptions are justifiable. But in the case of the cell fluid, which contains over 30 per cent of protein, the assumptions do not appear to be exact, as will be shown in a subsequent section on the determination of the dissociation constant of carbonic acid in cells.

EXPERIMENTAL METHODS.

Reduced and oxygenated defibrinated horse bloods were equilibrated at 38° with various CO₂ tensions. After centrifugation and transfer to small containers over mercury, determinations of the water, CO₂, Cl, and, where possible, electrometric estimations of the pH, of the serum and cells were made. The technique employed in saturation and separation of the serum and cells was that described by Van Slyke, Wu, and McLean (1). Reduction of the blood was obtained by two saturations with hydrogen.

Carbon dioxide and oxygen analyses of the blood were made in the manometric blood gas apparatus (11). Chloride determinations were made by oxidizing the protein of the serum and cells with concentrated nitric acid in the presence of known amounts of silver nitrate and titrating the excess silver according to the method recently described (3). Electrometric determinations of the hydrogen ion activity were made in the serum and cells of the reduced blood. Electrometric pH values of the oxygenated blood were impossible to obtain because of the effect of the oxygen on the hydrogen electrodes. All concentrations are expressed in millimols per kilo of water.

In all of the electrometric determinations the following procedure has been employed. The cell system used has been composed of

Saturated calomel cell	Saturated KCl bridge	Electrode liquid	H ₂ — Pt
------------------------------	----------------------------	---------------------	---------------------

A Clark-Cullen electrode vessel bearing a thermometer for the measurement of the temperature of the liquid contained the

solution whose reaction was to be measured. All measurements were performed in an air bath at a temperature of $38 \pm 0.10^\circ\text{C}$.

At the beginning of each experiment 0.1 N HCl was placed in the electrode vessel and the resulting potential measured. From this the ϵ of the calomel cell was calculated from the equation

$$\epsilon = \text{E.M.F.} - 1.08 \times 0.06169$$

1.08 is the value for pH of 0.1 N HCl calculated from Lewis and Randall.²

When a solution of unknown reaction is in the electrode vessel the pH of such a solution is calculated from the equation

$$\text{pH} = \frac{\text{E.M.F.} - \epsilon}{0.06169}$$

All potential measurements were corrected to 760 mm. of dry hydrogen (Clark³).

The calculation of pH values in this way involves three assumptions:

- (a) That the pH of 0.1 N HCl is 1.08 at 38°C .
- (b) That whatever diffusion potential may exist between the saturated KCl and the electrode liquid is the same in the case of 0.1 N HCl and the liquid of unknown reaction.
- (c) That the voltage developed in the hydrogen electrode would be the same in a serum or cell solution which had the same hydrogen ion activity as in the 0.1 N HCl solution with which our system was standardized.

None of the assumptions is certainly exact, but in the absence of more accurate information concerning the activity of ions of strong electrolytes, the magnitude of diffusion potentials, and the effect of serum and, particularly, cell constituents on ϵ we have utilized these assumptions in order to calculate pH values.

Determination of pK_1' of Carbonic Acid in Horse Serum.

The apparent dissociation constant K_1' in Henderson's equation represents the real constant K_1 divided by γ_{HCO_3} .

³ Clark (12), p. 459.

Hence the relationship between K_1' and K_1 may be used to estimate γ_{HCO_3} .

The apparent constant for serum was calculated from experimental data in Table III according to Hasselbalch's equation,

$$\text{p}K_1' = \text{pH} - \log \frac{[\text{HCO}_3]}{[\text{H}_2\text{CO}_3]}, \text{ where } \text{p}K_1' \text{ is } -\log K_1' \text{ and } [\text{HCO}_3]$$

TABLE III.

Determination of $\text{p}K_1'$ of Carbonic Acid and Calculation of $\text{p}\gamma_{\text{HCO}_3}$ in Horse Serum.

	[H ₂ O]	E.M.F.	Electrometric pH.	Total [CO ₂].	CO ₂ tension.	[H ₂ CO ₃]	[HCO ₃]	$\log \frac{[\text{HCO}_3]}{[\text{H}_2\text{CO}_3]}$	$\text{p}K_1'$	$\text{p}\gamma_{\text{HCO}_3}$
1924	$\frac{\text{gm.}}{\text{cc.}}$	mv.		$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	mm.	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$			
Feb. 23	0.937	706.5	7.61	30.67	30.1	0.982	29.69	1.480	6.13	0.20
	0.937	705.4	7.61	30.90	30.6	0.998	29.90	1.477	6.13	0.20
	0.937	693.9	7.41	32.87	50.2	1.636	31.23	1.281	6.13	0.20
	0.937	694.5	7.43	32.98	49.7	1.620	31.36	1.287	6.14	0.19
Jan. 17	0.933	670.8	7.07	46.64	151.2	4.93	41.71	0.927	6.14	0.19
	0.929	683.4	7.27	38.18	80.6	2.63	35.55	1.130	6.14	0.19
	0.928	692.6	7.42	30.18	43.8	1.43	28.75	1.303	6.12	0.21
Jan. 29	0.918	688.2	7.33	25.10	45.7	1.49	23.61	1.20	6.13	0.20
Mar. 13	0.932	673.3	7.10	47.20	15.50	5.06	42.14	0.921	6.18	0.15
	0.935	696.4	7.47	30.86	42.12	1.37	29.49	1.333	6.14	0.19
Mar. 20	0.934	670.9	7.06	45.22	150.4	4.90	40.32	0.914	6.15	0.18
	0.935	685.1	7.29	33.23	66.0	2.15	31.08	1.160	6.13	0.20
	0.932	699.4	7.52	23.57	26.1	0.85	22.72	1.426	6.09	0.24
Average.....									6.13	0.20

represents bicarbonate concentration. $\text{p}K_1'$ was found to be 6.13. Hastings and Sendroy (9) in a recent paper have determined the value for $\text{p}K_1$ to be 6.33. From these two figures one may estimate

$$\log \gamma_{\text{HCO}_3} = \text{p}K_1' - \text{p}K_1 = 6.13 - 6.33 = -0.20$$

$$\gamma_{\text{HCO}_3} = 0.63$$

Hastings and Sendroy have shown that the relationship between the bicarbonate activity coefficient and the ionic strength of bicarbonate-salt solutions is expressible as

$$-\log \gamma_{\text{HCO}_3'} = 0.5 \sqrt{\mu}$$

Substituting 0.2 for $-\log \gamma_{\text{HCO}_3'}$ one calculates $\mu = 0.160$, which agrees closely with the ionic strength of 0.167 estimated

TABLE IV.

Determination of pK_1' of Carbonic Acid in Reduced Horse Cells.

	$[\text{H}_2\text{O}]$	E.M.F.	Electrometric pH.	Total $[\text{CO}_2]$.	CO_2 tension.	$[\text{H}_2\text{CO}_3]$	$[\text{HCO}_3]$	$\frac{[\text{HCO}_3]}{\log [\text{H}_2\text{CO}_3]}$	pK_1'	$p\gamma_{\text{HCO}_3'}$
1924	$\frac{\text{gm.}}{\text{cc.}}$	mv.		$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	mm.	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$			
Jan. 17	0.661	657.0	6.84	42.90	151.2	4.930	37.97	0.887	5.95	0.38
	0.659	660.7	6.90	33.65	80.6	2.627	31.02	1.072	5.83	0.50
	0.656	673.7	7.12	25.62	43.8	1.430	24.19	1.228	5.89	0.44
Mar. 13	0.692	657.4	6.84	44.08	155.0	5.058	39.02	0.888	5.95	0.38
	0.693	675.7	7.14	26.34	42.1	1.372	24.97	1.260	5.88	0.45
Mar. 20	0.712	660.3	6.88	42.86	150.4	4.90	37.96	0.889	5.99	0.34
	0.699	675.4	7.12	31.46	66.0	2.15	29.31	1.133	5.99	0.34
	0.687	687.9	7.33	19.66	26.1	0.85	18.81	1.345	5.98	0.35
Nov. 30	0.711	664.1	6.97	38.9	112.5	3.67	35.2	0.98	5.99	0.34
	0.711	667.8	7.03	29.2	60.8	1.98	27.2	1.14	5.89	0.44
	0.711	676.3	7.16	19.2	27.4	0.90	18.3	1.31	5.85	0.48
Average.....									5.93	0.40

from serum analyses. It therefore appears probable that the three assumptions above stated, involved in the calculation of pH from potential readings, are valid for serum.

Determination of pK_1' of Carbonic Acid in the Cells of Horse Blood.

To determine the pK_1' of carbonic acid in undiluted cell fluid we have hemolyzed cells with saponin, then completely reduced the fluid by double or triple saturation with hydrogen containing sufficient CO_2 to give the desired pH. From the determined CO_2

tension, CO₂ content, and pH, the pK₁' values were calculated as $pK_1' = pH - \log \frac{[HCO_3]}{[H_2CO_3]}$. From eleven such experiments the pK₁' of carbonic acid in horse cells has been calculated to be 5.93 and $-\log \gamma_{HCO_3} = 0.40$. This would indicate γ_{HCO_3} to be 0.40 as contrasted with 0.62, the value calculated from the ionic strength of cell fluid. This discrepancy we are unable to

TABLE V.
Experiment to Determine $\frac{[\alpha_H]_s}{[\alpha_H]_c}$, $\frac{[Cl]_c}{[Cl]_s}$ and $\frac{[HCO_3]_c}{[HCO_3]_s}$ on Reduced Horse Blood.
Total [HbO₂] = 8.42 mm

No.		[H ₂ O]	CO ₂ tension.	[H ₂ CO ₃]	Total [CO ₂]	[HCO ₃]	$\log \frac{[HCO_3]}{[H_2CO_3]}$	pH	[Cl]	[HbO ₂]
		$\frac{gm.}{cc.}$	$mm.$	$\frac{mM}{kg. H_2O}$	$\frac{mM}{kg. H_2O}$	$\frac{mM}{kg. H_2O}$			$\frac{mM}{kg. H_2O}$	$\frac{mM}{kg. H_2O}$
1	Serum.	0.930	151.8	4.955	44.76	39.80	0.905	7.035	101.8	0.43
	Cells.	0.704	151.8	4.955	42.30	37.34	0.899	6.807	79.2	
2	Serum.	0.926	46.2	1.505	30.71	29.20	1.288	7.418	105.8	0.43
	Cells.	0.726	46.2	1.505	24.00	22.49	1.175	7.105	77.1	

	No. 1.	No. 2.
H _s	7.035	7.418
$\frac{[\alpha_H]_s}{[\alpha_H]_c}$	0.59	0.49
$\frac{[Cl]_c}{[Cl]_s}$	0.78	0.73
$\frac{[HCO_3]_c}{[HCO_3]_s}$	0.94	0.77

xplain. As stated above, Van Slyke, Wu, and McLean (1) assumed that the pK₁' value in the cell fluid was 6.12, the same as in serum, a conclusion to be expected from the approximate equality of the ionic strength in cells and serum, and from Warburg's (2) finding the same pK₁' in hemolyzed whole blood as in serum. Whether the actual pK₁' in cells is that estimated from the ionic strength, viz. 6.12 to 6.13, or that calculated from the electrometric pH measurements, we cannot at present decide.

It is possible that the high protein content of the cell fluid affects the potential readings in such a manner that an error is introduced in interpreting them into pH values by the usual formula. In the absence of further data which would make the calculation of

TABLE VI.

Experiment to Determine $\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$, $\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$, $\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$ for Reduced Horse Blood.

Total $[\text{HbO}_2] = 9.75 \text{ mM}$

No.		$[\text{H}_2\text{O}]$	CO_2 tension.	$[\text{H}_2\text{CO}_3]$	Total $[\text{CO}_2]$	$[\text{HCO}_3]$	$\frac{[\text{HCO}_3]}{\log [\text{H}_2\text{CO}_3]}$	pH	$[\text{Cl}]$	$[\text{HbO}_2]$
		$\frac{\text{gm.}}{\text{cc.}}$	mm.	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$				
1	Serum.	0.935	149.4	4.87	47.13	42.26	0.942	7.072	101.5	0.75
	Cells.	0.714	149.4	4.87	43.61	38.74	0.901	6.831	79.4	
2	Serum.	0.932	83.9	2.74	39.44	36.70	1.127	7.257	103.7	0.67
	Cells.	0.716	83.9	2.74	34.80	32.06	1.068	6.998	76.2	
3	Serum.	0.931	47.3	1.53	32.24	30.71	1.311	7.441	106.0	0.95
	Cells.	0.703	47.3	1.53	26.70	25.17	1.216	7.146	72.0	
4	Serum.	0.929	28.9	0.94	26.36	25.42	1.432	7.562	107.8	1.28
	Cells.	0.699	28.9	0.94	21.13	20.19	1.331	7.261	68.8	

	No. 1.	No. 2.	No. 3.	No. 4.
pH _s	7.072	7.257	7.441	7.562
$[\alpha_{\text{H}}]_s$	0.57	0.55	0.51	0.50
$[\alpha_{\text{H}}]_c$				
$[\text{Cl}]_c$	0.78	0.77	0.68	0.64
$[\text{Cl}]_s$				
$[\text{HCO}_3]_c$	0.92	0.87	0.82	0.79
$[\text{HCO}_3]_s$				

the activity coefficient of the bicarbonate ion in cells more certain we have used the value 5.93 for pK_1' in the calculation of the pH of oxygenated cells.

Determination of the Ratios $\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$, $\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$, $\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$

In Tables V to IX are given the results of five experiments on the distribution of α_{H} , Cl, and HCO_3 between serum and cells

TABLE VII.

Experiment to Determine $\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$, $\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$, $\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$ for Reduced Horse Blood.

Total $[\text{HbO}_2] = 9.61 \text{ mM}$

No.		$[\text{H}_2\text{O}]$	CO_2 tension.	$[\text{H}_2\text{CO}_3]$	Total $[\text{CO}_2]$	$[\text{HCO}_3]$	$\log \frac{[\text{HCO}_3]}{[\text{H}_2\text{CO}_3]}$	pH	$[\text{Cl}]$	$[\text{HbO}_2]$
		$\frac{\text{gm.}}{\text{cc.}}$	mm.	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$			$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$	$\frac{\text{mM}}{\text{kg. H}_2\text{O}}$
1	Serum.	0.934	151.2	4.930	46.64	41.71	0.928	7.058	101.9	1.09
	Cells.	0.661	151.2	4.930	42.90	37.97	0.887	6.817		
2	Serum.	0.929	80.6	2.627	38.18	35.55	1.132	7.262	103.2	1.30
	Cells.	0.659	80.6	2.627	33.65	31.02	1.072	7.002		
3	Serum.	0.928	43.8	1.43	30.18	28.75	1.303	7.433	102.4	
	Cells.	0.656	43.8	1.43	25.62	24.19	1.288	7.158		
4	Serum.	0.943	29.8	0.97	26.90	25.93	1.427	7.557	107.8	1.34
	Cells.	0.648	29.8	0.97	22.20	21.23	1.340	7.270		

	No. 1.	No. 2.	No. 3.	No. 4.
$[\text{I}_s]$	7.058	7.262	7.433	7.557
$[\text{I}]_s$	0.52	0.55	0.53	0.52
$[\text{I}]_c$				
$[\text{I}]_c$		0.71		0.62
$[\text{I}]_s$				
$[\text{CO}_3]_c$	0.91	0.87	0.84	0.82
$[\text{CO}_3]_s$				

affected by changes in pH_s from 7.0 to 7.6. Two experiments (Tables VIII and IX) include data showing the effect of oxygenation and reduction on the distribution of these ions. The pH

values given are calculated from Equation 5 and the pK_1' value given in Tables III and IV; viz., 6.13 for serum and 5.93 for cells

TABLE VIII.

Experiment to Determine $\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$, $\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$, $\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$ in Oxygenated and Reduced Horse Blood.

Total $[\text{HbO}_2] = 9.37 \text{ mm}$

No.		$\frac{[\text{H}_2\text{O}]}{\text{gm. cc.}}$	CO_2 tension. mm.	$\frac{[\text{H}_2\text{CO}_3]}{\text{mM kg. H}_2\text{O}}$	$\frac{\text{Total } [\text{CO}_2]}{\text{mM kg. H}_2\text{O}}$	$\frac{[\text{HCO}_3]}{\text{mM kg. H}_2\text{O}}$	$\frac{[\text{HCO}_3]}{\log \frac{[\text{H}_2\text{CO}_3]}{[\text{HCO}_3]}}$	pH	$\frac{[\text{Cl}]}{\text{mM kg. H}_2\text{O}}$	$\frac{[\text{HbO}_2]}{\text{mM kg. H}_2\text{O}}$
1	Reduced, serum.	0.932	155.0	5.058	47.20	42.14	0.921	7.051	106.5	0.23
	Reduced, cells.	0.692	155.0	5.058	44.08	39.02	0.888	6.818	85.4	
2	Reduced, serum.	0.935	42.1	1.372	30.86	29.49	1.333	7.463	112.1	0.83
	Reduced, cells.	0.693	42.1	1.372	26.34	24.97	1.260	7.190	74.8	
3	Oxygenated, serum.	0.936	39.3	1.284	27.63	26.35	1.313	7.443	114.3	9.04
	Oxygenated, cells.	0.668	39.3	1.284	20.13	18.85	1.168	7.098	69.8	

	No. 1, reduced.	No. 2, reduced.	No. 3, oxygenated.
pH_s	7.051	7.463	7.443
$\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$	0.58	0.53	0.45
$\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$	0.80	0.67	0.60
$\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$	0.93	0.85	0.72

The $[\text{H}_2\text{CO}_3]$ molality in millimols per kilo of water in both serum and cells is calculated from the equation

$$(6) \quad [\text{H}_2\text{CO}_3] = \frac{P_{\text{CO}_2} \times 0.555}{760 \times 0.0224} = 0.0326 P_{\text{CO}_2}$$

TABLE IX.

Experiment to Determine $\frac{[\alpha_H]_e}{[\alpha_H]_e}, \frac{[Cl]_e}{[Cl]_e}, \frac{[HCO_3]_e}{[HCO_3]_e}$ in Oxygenated and Reduced Horse Blood.

Total [HbO₂] = 8.76 mm

No.		[H ₂ O]	CO ₂ tension.	[H ₂ CO ₃]	Total CO ₂ .	[HCO ₃]	$\log \frac{[HCO_3]}{[H_2CO_3]}$	pH	[Cl]	[HbO ₂]
		$\frac{gm.}{cc.}$	mm.	$\frac{mM}{kg. H_2O}$	$\frac{mM}{kg. H_2O}$	$\frac{mM}{kg. H_2O}$			$\frac{mM}{kg. H_2O}$	$\frac{mM}{kg. H_2O}$
1	(Reduced).									
	Serum.	0.934	150.4	4.90	45.22	40.32	0.914	7.044	101.4	0.34
	Cells.	0.712	150.4	4.90	42.86	37.96	0.889	6.819	83.0	
2	Serum.	0.935	66.0	2.15	33.23	31.08	1.160	7.290	105.2	0.49
	Cells.	0.699	66.0	2.15	31.46	29.31	1.133	7.063	74.3	
3	Serum.	0.932	26.1	0.851	23.57	22.72	1.426	7.556	107.2	1.21
	Cells.	0.687	26.1	0.851	19.66	18.81	1.345	7.275	69.6	
4	(Oxygen- ated).									
	Serum.	0.927	149.4	4.87	41.95	37.08	0.881	7.011	102.8	8.70
	Cells.	0.651	149.4	4.87	38.45	33.58	0.839	6.769	76.8	
5	Serum.	0.933	56.5	1.84	29.78	27.94	1.181	7.311	106.7	7.96
	Cells.	0.655	56.5	1.84	24.26	22.42	1.085	7.015	66.8	
6	Serum.	0.934	24.3	0.79	21.29	20.50	1.414	7.544	110.2	8.72
	Cells.	0.635	24.3	0.79	15.12	14.33	1.258	7.188	62.7	

	No. 1, reduced.	No. 2, reduced.	No. 3, reduced.	No. 4, oxygenated.	No. 5, oxygenated.	No. 6, oxygenated.
H ₂	7.044	7.290	7.556	7.011	7.311	7.544
$\frac{[H]_e}{[H]_e}$	0.60	0.59	0.52	0.57	0.51	0.44
$\frac{[Cl]_e}{[Cl]_e}$	0.82	0.71	0.65	0.75	0.63	0.57
$\frac{[HCO_3]_e}{[HCO_3]_e}$	0.94	[0.94]	0.83	0.91	0.80	0.71

From determinations of the solubility of CO_2 in acidified serum and in dilute acidified cell solutions it was found that within the limits of our analytical precision, the other solutes have no effect on the solubility of CO_2 per kilo of blood water. The value of the molality $[\text{H}_2\text{CO}_3]$ thus calculated is also the value for the activity $\alpha_{\text{H}_2\text{CO}_3}$.

The $[\text{HCO}_3]$ was obtained by subtracting the $[\text{H}_2\text{CO}_3]$ concentration from the total $[\text{CO}_2]$ content.

With these data we have calculated the ratios $\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$, $\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$ and $\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$ for oxygenated and reduced blood from pH 7 to 7.6

TABLE X.

Summary of Experimental Distribution of H^+ , Cl , and HCO_3 between Serum and Cells.

pH _s	H ⁺ activity, Cl molality, and HCO ₃ molality ratios.					
	Reduced blood.			Oxygenated blood.		
	$\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$	$\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$	$\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$	$\frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c}$	$\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$	$\frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$
7.0	0.60	0.81	0.94	0.57	0.74	0.89
7.2	0.57	0.75	0.89	0.52	0.68	0.83
7.4	0.53	0.68	0.85	0.47	0.61	0.77
7.6	0.49	0.62	0.80	0.42	0.54	0.71

These values have been plotted in Figs. 1 to 3 and the interpolated mean values are tabulated in Table X.

Determined ratios have been calculated to 100 per cent oxygenation and reduction by making the following slight correction. The difference between the activity ratios for oxygenated and reduced blood between pH 7 and 7.6 was taken to be a linear function of the pH, approximately represented by the equation

$$r_R - r_O = 0.1 (\text{pH} - 6.6)$$

The correction, c , applied to the ratio was

$$c = (r_R - r_O) \times \text{percentage saturation.}$$

Since the correction was in all cases extremely small, no significant error is introduced by the assumption that difference in the activity ratio is a linear function of the pH and of the percentage saturation of the blood with oxygen.

DISCUSSION OF THE DETERMINED DISTRIBUTION RATIOS.

The results, summarized in Figs. 1, 2, and 3, show the following.

(1) The present $[\text{HCO}_3]$ concentration ratios (Fig. 1) follow the r curves calculated theoretically by Van Slyke, Wu, and McLean for the blood of their horse with about the same degree

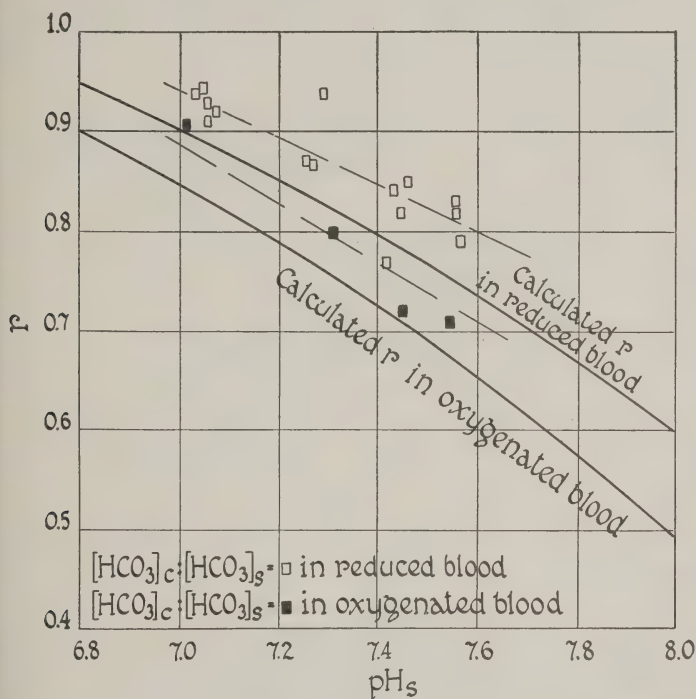


Fig. 1. Points designated □ indicate determined ratios of bicarbonate total concentrations in cells to bicarbonate molalities in serum at different H_2 values of reduced blood. Points designated ■ were obtained from oxygenated blood. The solid curves were plotted from Equation 1.

of approximation as their $[\text{HCO}_3]$ ratios. The present ratios lie above the calculated r curves whereas theirs lay below. The methods used were the same. Consequently the difference in experimental $[\text{HCO}_3]$ ratios is presumably due to the fact that the blood used in our experiments differs somewhat in protein or alkali content from theirs. We did not perform total base and protein analyses.

(2) The $[\text{Cl}]_c:[\text{Cl}]_s$ concentration ratios (Fig. 2) are consistently lower than the $[\text{HCO}_3]_c:[\text{HCO}_3]_s$ ratios. Van Slyke, Wu, and McLean found no consistent difference between HCO_3 and Cl ratios, but stated that the absolute values of their Cl ratios were uncertain, because the Cl method then used was not of tested reliability for concentrated cell substance. We believe that the method here used (3) is reliable, and that the results indicate a genuine difference between the HCO_3 and the Cl molal

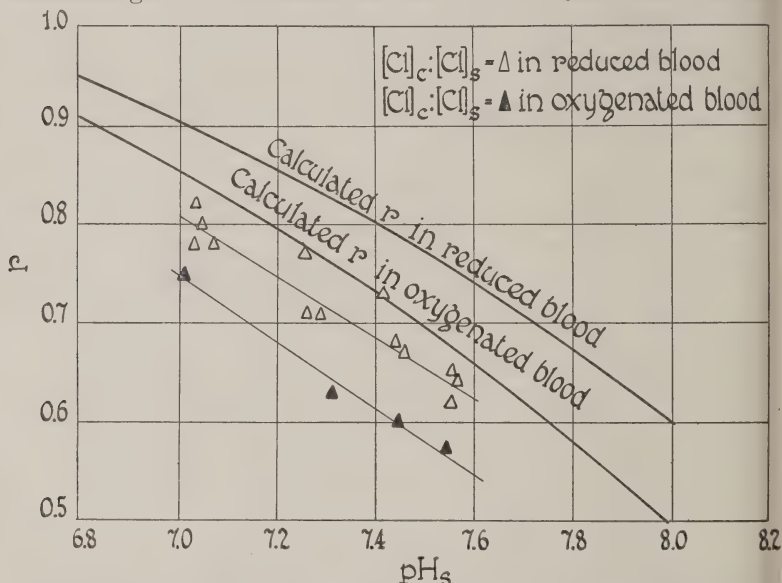


FIG. 2. Points designated Δ indicate determined ratios of chloride molal concentrations in cells to chloride molalities in serum at different pH_s values of reduced blood. Points designated \blacktriangle were obtained from oxygenated blood. The calculated r curves were plotted from Equation 1.

ratios. Instead of having $\frac{[\text{Cl}]_c}{[\text{Cl}]_s} = \frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$ we have on the average $\frac{[\text{Cl}]_c}{[\text{Cl}]_s} = 0.81 \frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$.

(3) The hydron activity values, α_{H^+} and $\alpha_{\text{H}_2\text{CO}_3}$, were determined by direct electrometric measurement in reduced blood. In oxygenated blood the $\alpha_{\text{H}_2\text{CO}_3}$ values were estimated by calculation with Hasselbalch's equation $-\log \alpha_{\text{H}^+} = \text{pK}_1' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$.

The value used for pK_1' , 5.93, was based on electrometric measurements in cell fluids, so that the calculated α_{H_c} values are equivalent to electrometric ones. The activity distribution ratio, $\alpha_{H_s}:\alpha_{H_c}$, showed lower values than the molal $[Cl]_c:[Cl]_s$ and $HCO_3]_c:[HCO_3]_s$ distribution ratios. The relationship between this fact and the presumable inequality of the activity coefficients in cells and serum will be discussed shortly.

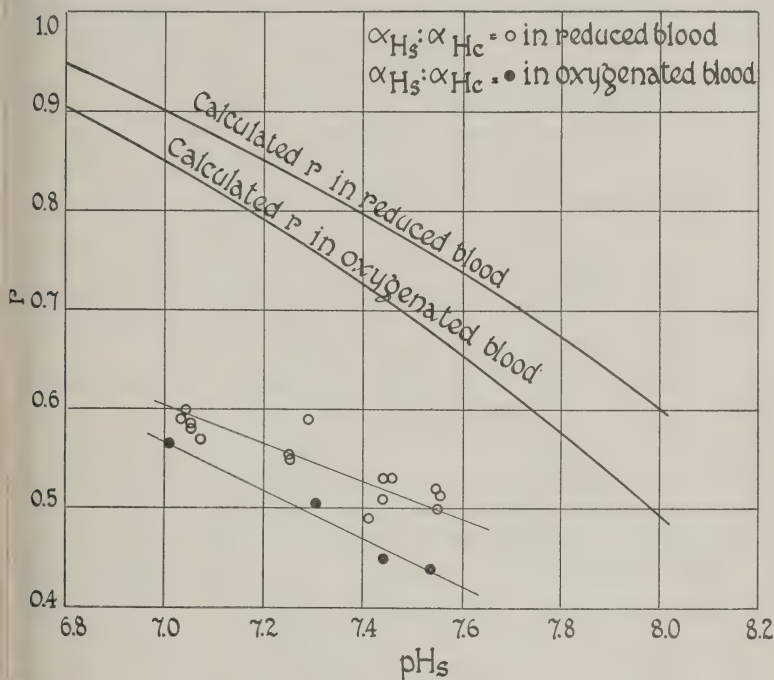


FIG. 3. Points designated \circ indicate determined ratios of hydrogen on activity in serum to hydrogen ion activity in cells at different pH_s values in reduced blood. Points designated \bullet were obtained upon oxygenated blood. The calculated r curves were plotted from Equation 1.

(4) The $[Cl]$ and $[HCO_3]$ molal distribution ratios under the influence of changing pH and oxygenation follow curves which parallel the molal r curves theoretically estimated by Van Slyke, Vu, and McLean, in their Equations 10 and 14, from the base-binding powers of the proteins (Figs. 1 and 2). The observed molal HCO_3 and Cl distribution curves run at definitely different

levels from the calculated r curves, the experimental $[\text{HCO}_3]$ ratios lying above the theoretical curves, the $[\text{Cl}]$ ratios below them. The differences in levels seem not greater than might be expected from the possible effects of the cell and serum constituents on the activities of the two anions. The electrometrically determined H^+ activity distribution ratios show a similar parallelism with the calculated r curves, although at a much lower level (Fig. 3). The consistent parallelism of observed and calculated effects of both CO_2 and O_2 tension changes on all three ratios affords evidence of the probable adequacy of the physicochemical laws used to explain the distribution of the diffusible ions as a function of the base bound by the proteins, provided the factors are included which affect the activities of the ions in serum and cells.

Relative Activity Ratios of H^+ , Cl' , and HCO_3' in Cells and Serum.

The relationship of our experimentally determined distribution ratios of α_{H^+} , $[\text{HCO}_3]$, and $[\text{Cl}]$ may be indicated as follows:

$$(7) \quad \frac{[\alpha_{\text{H}^+}]_c}{[\alpha_{\text{H}^+}]_s} = 0.77 \frac{[\text{Cl}]_c}{[\text{Cl}]_s} = 0.62 \frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s}$$

The Cl and HCO_3 concentrations have been determined by accurate chemical analyses, and appear to be trustworthy. It is possible that the high protein content of the cells has affected the E.M.F. readings from which the α_{H^+} values are estimated. However, we have no independent evidence of such an effect, and the $\alpha_{\text{H}^+}_s : \alpha_{\text{H}^+}_c$ ratio is the only one of the three based on methods which theoretically give directly activity values. Granted the correctness of the α_{H^+} values, and of Donnan's law for the thermodynamic activity distribution of the diffusible ions, there must be lower activity coefficients for Cl' and HCO_3' in the cells than in the serum.

This difference necessitates the introduction of activity coefficients into Equations 10 and 14 of Van Slyke, Wu, and McLean, in order to render them exact in indicating activity distributions. It does not, however, affect the validity of the equations in their original form for showing the relationship of the mean *molal* distribution ratios of diffusible anions between cells and serum.

The approximate equality of the *osmotic* activity coefficients of the electrolytes in cells and serum respectively was demonstrated by the chemical analyses of cells and serum reported in Table I of the paper by Van Slyke, Wu, and McLean. It is not at all inconsistent with inequality of the thermodynamic activity coefficients. Assuming equality of the osmotic activity coefficients we may express the osmolar activities of the quantitatively important constituents of cells and serum as follows (see Equation 6 of Van Slyke, Wu, and McLean):

$$(8) \quad \begin{aligned} 2[\text{BCl}]_c + 2[\text{BHCO}_3]_c + [\text{BP}]_c + [\text{Hb}]_c &= 2[\text{BCl}]_s + \\ 2[\text{BHCO}_3]_s + [\text{BP}]_s \end{aligned}$$

Dividing through by $2([\text{BCl}]_s + [\text{BHCO}_3]_s)$ we obtain

$$(9) \quad \frac{[\text{BCl}]_c + [\text{BHCO}_3]_c}{[\text{BCl}]_s + [\text{BHCO}_3]_s} = 1 - \frac{[\text{BP}]_c - [\text{BP}]_s + [\text{Hb}]_c}{2([\text{BCl}]_s + [\text{BHCO}_3]_s)} = r_{\text{molal}}$$

This is equivalent to Equation 8 of Van Slyke, Wu, and McLean. In this equation no assumption enters of Donnan's activity distribution: the only assumptions concern osmotic pressures, and the conclusion indicated is that the *sum of chloride and bicarbonate* molalities in the cells is related to their sum in the serum as indicated by the right-hand member of the equation. If the Cl molal ratio is greater than the $[\text{Cl} + \text{HCO}_3]$ molal ratio the $[\text{HCO}_3]$ ratio must be less than the latter, and *vice versa*. Such a relation is not excluded. It is in fact the one shown by our present Cl and HCO_3 analyses.

In order to conform with Donnan's law, however, the thermodynamic activity ratios of the diffusible ions must be equal.

$$(10) \quad \frac{[\alpha_{\text{Cl}}]_c}{[\alpha_{\text{Cl}}]_s} = \frac{[\alpha_{\text{HCO}_3}]_c}{[\alpha_{\text{HCO}_3}]_s} = \frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c} = r_{\text{activity}}$$

Introducing the activity coefficients to indicate the relationship between molality and thermodynamic activity the above equation becomes

$$(11) \quad \frac{\gamma_{\text{Cl}_c} [\text{Cl}]_c}{\gamma_{\text{Cl}_s} [\text{Cl}]_s} = \frac{\gamma_{\text{HCO}_3c} [\text{HCO}_3]_c}{\gamma_{\text{HCO}_3s} [\text{HCO}_3]_s} = \frac{[\alpha_{\text{H}}]_s}{[\alpha_{\text{H}}]_c} = r_{\text{activity}}$$

where $[\text{Cl}]_c$ etc. represent molal concentrations and γ_{Cl_c} etc. the activity coefficients by which the molalities are multiplied to give thermodynamic activities.

Comparison of Equations 9 and 11 shows that the mean molal distribution ratio r_{molal} of diffusible ions between cells and serum can equal the activity distribution ratio r_{activity} only if the activity coefficient of each diffusible ion is the same in cell

and serum; that is, if $\frac{\gamma_{\text{HCO}_3c}}{\gamma_{\text{HCO}_3s}} = 1$, etc. Van Slyke, Wu, and

McLean, as a preliminary approximation, assumed the existence of this condition. Our present results indicate that the ratios $\gamma_{\text{HCO}_3c}:\gamma_{\text{HCO}_3s}$ and $\gamma_{\text{Cl}_c}:\gamma_{\text{Cl}_s}$ differ measurably from each other and that if the electrometric $[\alpha_{\text{H}}]$ ratios are correct, the γ value for both Cl and HCO_3 are lower in cells than serum.

In Table X are given the experimental data from which are

calculated the $\frac{\gamma_{\text{HCO}_3c}}{\gamma_{\text{HCO}_3s}}$ and $\frac{\gamma_{\text{Cl}_c}}{\gamma_{\text{Cl}_s}}$ values that, when inserted into

Equation 11, cause the Cl' and HCO_3' activity distribution ratio thus estimated from the molal ratios, to equal the α_{H} distribution ratios. The $[\text{Cl}]_c:[\text{Cl}]_s$, $[\text{HCO}_3]_c:[\text{HCO}_3]_s$, and $[\alpha_{\text{H}}]_c:[\alpha_{\text{H}}]_s$ values in Table X are obtained by interpolation on the curve representing the mean experimental values in Figs. 1, 2, and 3.

The value for $\frac{\gamma[\text{Cl}]_c}{\gamma[\text{Cl}]_s}$ is obtained by dividing each $\frac{[\alpha_{\text{H}}]_c}{[\alpha_{\text{H}}]_s}$ value by

the $\frac{[\text{Cl}]_c}{[\text{Cl}]_s}$ value for the same pH and degree of oxygenation. The

eight $\frac{\gamma[\text{Cl}]_c}{\gamma[\text{Cl}]_s}$ values thus obtained ranged from 0.74 to 0.79, and

averaged 0.77. Similarly the $\frac{\gamma_{\text{HCO}_3c}}{\gamma_{\text{HCO}_3s}}$ values were obtained ranging

from 0.59 to 0.64 and averaging 0.62. Equation 11, with these factors inserted, becomes

$$(12) \quad \frac{\alpha_{\text{H}_s}}{\alpha_{\text{H}_c}} = 0.77 \frac{[\text{Cl}]_c}{[\text{Cl}]_s} = 0.62 \frac{[\text{HCO}_3]_c}{[\text{HCO}_3]_s} = r_{\text{activity}}$$

That consistency is introduced among the experimentally obtained distribution ratios by introducing these factors for

calculating activity ratios from molal ratios, is demonstrated by the data in Tables XI.

If the electrometric pH determinations in cell contents are exact and Donnan's law holds for the distribution of diffusible ions the above γ ratios seem valid. If the cell α_H values should be corrected later, the γ ratios would require multiplication by the same factor and likewise the $r_{activity}$ values, but the soundness of the theoretical considerations on which the latter are based would not be affected, nor the relative proportions of the $\frac{\gamma_{HCO_3c}}{\gamma_{HCO_3s}}$

TABLE XI.
Activity Ratios.

α_H ratios determined by E.M.F. measurement.

$\alpha_{Cl'}$ and $\alpha_{HCO_3'}$ ratios calculated from Cl and HCO_3 ratios by arbitrary activity factors.

pH _s	Reduced blood.			Oxygenated blood.		
	$\frac{[\alpha_{H'}]_s^*}{[\alpha_{H'}]_c}$	$\frac{[\alpha_{Cl'}]_c^\dagger}{[\alpha_{Cl'}]_s}$	$\frac{[\alpha_{HCO_3'}]_c^\ddagger}{[\alpha_{HCO_3'}]_s}$	$\frac{[\alpha_{H'}]_s^*}{[\alpha_{H'}]_c}$	$\frac{[\alpha_{Cl'}]_c^\dagger}{[\alpha_{Cl'}]_s}$	$\frac{[\alpha_{HCO_3'}]_c^\ddagger}{[\alpha_{HCO_3'}]_s}$
7.0	0.60	0.62	0.58	0.57	0.57	0.55
7.2	0.57	0.58	0.55	0.52	0.52	0.51
7.9	0.53	0.52	0.53	0.47	0.47	0.48
7.6	0.49	0.48	0.50	0.42	0.42	0.44

* From pH values directly determined with gas chain.

† Calculated as $\frac{[\alpha_{Cl'}]_c}{[\alpha_{Cl'}]_s} = \frac{[\gamma_{Cl}]_c}{[\gamma_{Cl}]_s} \frac{[Cl]_c}{[Cl]_s}$ with $\frac{[\gamma_{Cl}]_c}{[\gamma_{Cl}]_s} = 0.77$

‡ Calculated as $\frac{[\alpha_{CO_3'}]_c}{[\alpha_{HCO_3'}]_s} = \frac{[\gamma_{HCO_3}]_c}{[\gamma_{HCO_3}]_s} \frac{[HCO_3]_c}{[HCO_3]_s}$ with $\frac{[\gamma_{HCO_3}]_c}{[\gamma_{HCO_3}]_s} = 0.625$

and $\frac{\gamma_{Clc}}{\gamma_{Cls}}$ ratios. Thus, if the H' activities should be calculated by Henderson's equation with the same K_1' values for cells as for serum, the relationships would be expressed as

$$\frac{[\alpha_{H'}]_s}{[\alpha_{H'}]_c} = \frac{[HCO_3]_c}{[HCO_3]_s} = 0.81 \frac{[Cl]_c}{[Cl]_s}$$

We have attempted to obtain independent measurements of the Cl activity coefficients in cells and serum by electrometric

α_{Cl} determinations, but the conditions in both cells and serum have thus far prevented the attainment of the necessary accuracy

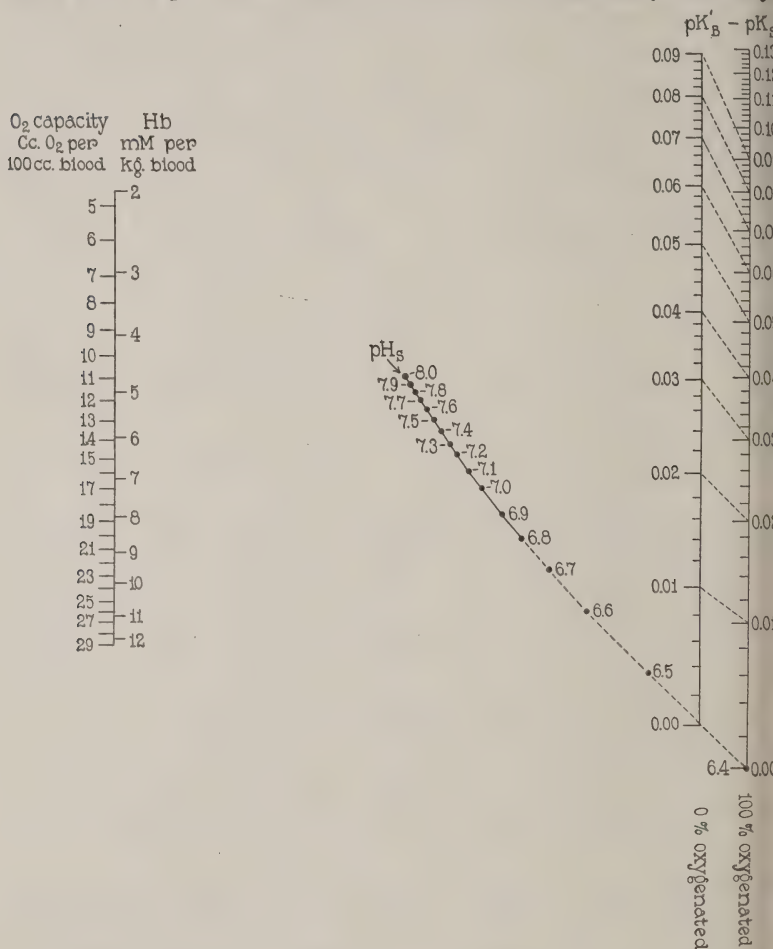


FIG. 4.

Value of pK_1' in Oxygenated and Reduced Whole Blood.

Van Slyke, Wu, and McLean (1) estimated the effect which inequality in HCO_3 distribution has on the pK_1' of Hasselbalch's equation when it is applied to oxygenated whole blood to determine plasma pH. They did not extend their calculation to

reduced blood, as the quantitative effect of oxygenation and reduction on the ionic distribution had not then been determined. With the present data in hand such an extension appears now justified, and the results are incorporated in Fig. 4, which is a d'Ocagne nomogram similar to Fig. 6, *b* of Van Slyke, Wu and McLean, with the addition of a line for the $pK_b' - pK_s'$ values for reduced blood. The dashed slanting lines are to assist in interpolations when pK_1' values are estimated for partially reduced blood.

SUMMARY.

1. The distribution of the diffusible ions, H^+ , Cl' , and HCO_3' between serum and cells of horse blood has been studied over the pH range 7.0 to 7.6, and in oxygenated and reduced blood.
2. The experimentally determined distribution ratios have been found to be related as follows:

$$\frac{[\alpha_{H^+}]_c}{[\alpha_{H^+}]_s} = 0.77 \frac{[Cl]_c}{[Cl]_s} = 0.62 \frac{[HCO_3]_c}{[HCO_3]_s}$$

α_{H^+} represents hydron activity electrometrically determined and $[Cl]$ and $[HCO_3]$ represent molalities, in terms of mols of chloride and bicarbonate per kilo of water.

3. The activity coefficient of HCO_3 in serum estimated from determined $[H_2CO_3]$, $[BHCO_3]$, and pH values was found to be related to the ionic strength in the manner predicted for salt solutions by the theory of Debye and Hückel. For cells, however, the ionic strength as calculated by us indicated a much greater activity coefficient than that found. It appears that in cells unknown factors influence either the activity coefficients or the potential readings obtained with the hydrogen electrode.

4. The changes in the distribution of α_{H^+} , Cl , and HCO_3 between serum and cells with change in serum pH and in degree of oxygenation of the hemoglobin approximate those predicted by Van Slyke, Wu, and McLean, from the changes in base-binding power of the cell and serum proteins caused by varying pH and oxygenation.

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THE INFLUENCE OF PROTEIN CONCENTRATION ON THE CONDUCTIVITY OF HUMAN SERUM.

BY DANA W. ATCHLEY AND EMILY G. NICHOLS.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York.)

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INTRODUCTION.

A few years ago it was found^{1,2} that the effect of pure gelatin and crystalline egg albumin on the conductivity of a 0.6 per cent NaCl solution is dependent on the hydrogen ion concentration. Additional increments of protein increased total conductivity when the proteins were highly ionized (pH 3.3) and decreased it when they were relatively less ionized (pH 5.1 and 7.4). At the reaction of human serum (pH 7.4), where the proteins are but slightly ionized, the conductivity decreases as the concentration of serum protein increases, providing, of course, that the other electrolytes remain constant.

In 1895, Sjöqvist³ in the course of a long series of experiments on the physiological chemistry of hydrochloric acid, added egg albumin in varying amounts to a 0.05 N NaCl solution, determining thereafter the conductivities of these solutions. There was very little change as the albumin percentage increased; for example, the molecular conductivity of the pure salt solution was 89.6, whereas that of the 6.3 per cent albumin solution was 89.2. It is probable that these experiments were carried on at a pH just between the point where relatively high protein ionization causes an increase of total conductivity and the lower level of ionization where the protein actually decreases conductivity.

¹ Palmer, W. W., Atchley, D. W., and Loeb, R. F., *J. Gen. Physiol.*, 1920-21, iii, 801.

² Palmer, W. W., Atchley, D. W., and Loeb, R. F., *J. Gen. Physiol.*, 1921-22, iv, 585.

³ Sjöqvist, J., *Skand. Arch. Physiol.*, 1895, v, 332.

This suggestion is supported by Sjöqvist's statement that he added HCl to his egg albumin during its preparation.

3 years later, 1898, Bugarszky and Tangl⁴ attempted to determine the factor of correction required in order to compare the conductivities of sera varying in their protein content. These authors dialyzed human serum against distilled water for about 2 months, concentrated at reduced pressure, and added the resulting protein in increasing amounts to salt solutions (containing electrolytes in relative proportion as they occur in blood) of equal conductivity. The pH was not controlled. The conductivity of a similar concentration of protein in distilled water was also observed in each instance. A factor was obtained by calculating the decrease (K) in conductivity for each gram of protein (per 100 cc.), according to the following formula.

$$(1) \quad K = \frac{C_s - (C_m - C_p)}{C_s} \cdot \frac{100}{P}$$

C_s = conductivity of salt solution.

C_m = conductivity of salt solution plus protein.

C_p = conductivity of protein in distilled water.

P = percentage of serum protein.

The results as determined ranged from 1.82 to 2.90, averaging 2.50. It seems clear, however, that the above formula does not develop the required factor, for it represents only the interference of the protein with conductivity rather than the total change in conductivity upon the addition of protein to salt solution. This total change is presumably the resultant of two effects:

(1) Increase in conductivity by the protein ions. (2) Decrease in conductivity by the unionized protein. This resultant correction may be determined by the simple formula

$$(2) \quad K = \frac{C_s - C_m}{C_s} \cdot \frac{100}{P}$$

According to formula (2) Bugarszky and Tangl's results average 1.36 instead of 2.50. Two of their experiments are plotted in Fig. 1 (Curves E and F), where the curves are seen to be roughly

⁴ Bugarszky, S., and Tangl, F., *Arch. ges. Physiol.*, 1898, lxxii, 531.

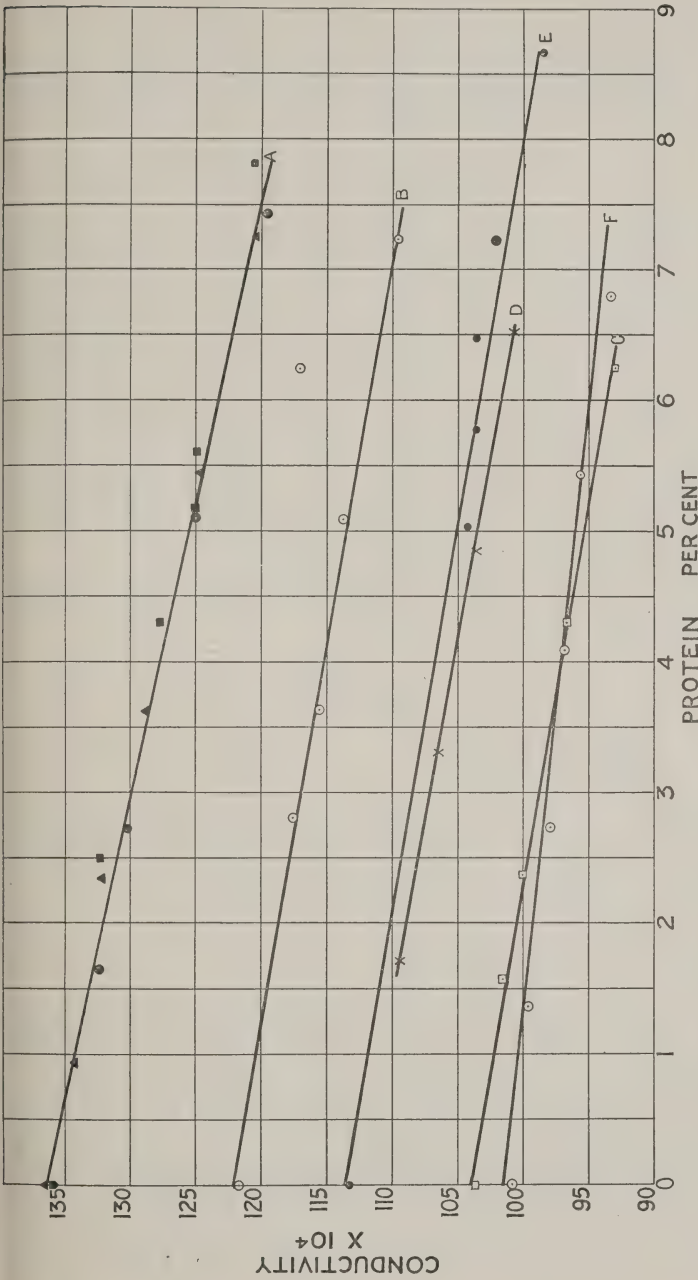


FIG. 1. The relation of the conductivity $\times 10^4$ (ordinates) to the percentage of serum protein (abscissae) in human serum (Curves A, B, and C) and gelatin (Curve D). Curves E and F are derived from two experiments of Bugarszky and Tangl.

parallel to those obtained by us. More exact agreement is not to be expected without control of hydrogen ion concentration.

More recently Gram and Cullen⁵ have worked on this problem and find $K = 2.2$. Their formula is

$$(3) \quad K = (C_s + C_p - C_m) \cdot \frac{100}{P}$$

Here again, the conductivity of the protein itself is introduced, and these authors further modify the formula by correcting back to the protein solution rather than to the salt solution. It would seem somewhat more practical to relate the actual serum conductivity to a non-protein solution of the same electrolyte content, as is done in formulas (1) and (2). If Gram and Cullen's experiments (Table VIII⁵) are recalculated according to formula (2) their average for K becomes 1.57. These authors prepared their serum protein by acidifying, dialyzing against distilled water, titrating back to pH 7.4, and redialyzing against distilled water. They determined protein refractometrically.

In both the above papers^{4, 5} the final correction of the observed conductivity was made by the formula

$$(4) \quad C_c = C_o \frac{100}{100 - (K \times p)}$$

C_c = corrected conductivity.

C_o = observed conductivity.

It is obvious that neither the factor K nor formula (4) will serve when the conductivity of the non-protein electrolytes falls below that of the protein itself. This, however, is unimportant in the present discussion, because there is relatively little variation in the salt content of human serum.

The experiments just reviewed have the common fault that the protein during purification suffered considerable change in pH and salt concentration. The present paper describes a method of preparing serum protein solutions of equal electrolyte content, yet at the same time avoiding significant physical or chemical alteration. With such solutions the factor K has again been determined and found to agree surprisingly well with the values obtained by Bugarszky and Tangl and Gram and Cullen.

⁵ Gram, H. C., and Cullen, G. E., *J. Biol. Chem.*, 1923, lvii, 477.

EXPERIMENTAL.

Blood was obtained at therapeutic phlebotomy with oiled (albolene) syringes, allowed to clot, and the serum separated by centrifuging—no hemolysis occurred.

TABLE I.

Experiment No.	Protein.	Cl'mm per liter	pH	Conductivity x 10 ⁴
	<i>per cent</i>			
1	1 0	128.8	7.7	135.8
	2 2.5	128.7		132.3
	3 4.3	127.9	7.3	127.9
	4 5.6	127.9	7.5	125.0
	5 7.8	127.5	7.6	120.6
2	1 0	128.9	7.7	136.5
	2 0.9	129.2	7.3	134.4
	3 2.3	126.5	7.4	132.2
	4 3.6	126.5	7.6	128.8
	5 5.4	124.5	7.6	124.7
	6 7.3	123.1	7.7	120.4
3	1 0	128.3	7.3	135.6
	2 1.7	128.3	7.4	132.3
	3 2.7	127.3	7.4	130.2
	4 5.1	127.9	7.5	125.0
	5 7.4	127.5	7.5	119.6
4	1 0	114.7	7.6	121.8
	2 2.8	113.5	7.5	117.7
	3 3.6	113.5	7.6	115.7
	4 5.1	113.5	7.6	113.7
	5 7.2	114.7	7.7	109.6
5	1 0	96.1	7.4	103.9
	2 1.6	98.8	7.4	101.7
	3 2.4	98.8	7.4	100.3
	4 4.3	97.8	7.5	96.9
	5 6.2	96.1	7.4	93.1

About 100 cc. of this serum were dialyzed for 3 or 4 days against 12 liters of 0.8 per cent NaCl solution, brought to pH 7.4 by means of a small amount of 0.1 N NaH CO₃. The dialyzate was changed daily. The serum remained clear throughout, and at the end of dialysis tests for non-protein nitrogen were negative. The Cl' content of the dialyzed serum was then

determined by analysis and the pH colorimetrically. A salt solution of the same Cl' content was prepared and brought to pH 7.4 with 0.1 N NaHCO_3 . There were thus produced two similar salt solutions, one of which contained serum protein. There were equal amounts of the electrolyte of greater concentration (NaCl), and practically equal amounts in very low concentration, ≈ 0.0005 N, of the only other electrolyte (NaHCO_3) present. The conductivities (at $25^\circ\text{C}.$) of the salt solution, the serum, and mixtures of the two, gave a series of observations on solutions of equal electrolyte content but with varying percentages of unchanged serum protein. The pH, Cl', and protein percent (Kjeldahl) were determined and the results are recorded in Table I and charted in Fig. 1. Three experiments were performed at a Cl' level, such that the conductivity of the strongest serum solution was that of normal human serum; two additional experiments at lower conductivities show practically parallel results.

DISCUSSION.

Curves A, B, and C, Fig. 1, chart the results obtained by us; the various points determined fall quite accurately on the lines, and the three curves are approximately parallel. The slope of Curve D, which represents the experiments with gelatin mentioned above⁴ is not inconsistent with those for blood serum. The factor K has been determined by formula (2) for each curve and is

$$\begin{aligned}\text{Serum, Curve A} &= 1.58 \text{ (three experiments).} \\ \text{Curve B} &= 1.39 \text{ (one experiment).} \\ \text{Curve C} &= 1.62 \text{ (one experiment).} \\ \text{Gelatin, Curve D} &= 1.65.\end{aligned}$$

The average value of K (for serum) is 1.55. By the same formula, it will be remembered, Bugarszky and Tangl obtained 1.36, and Gram and Cullen 1.57. Since the composite curve of three experiments at conductivities closest to normal values for human serum gave $K = 1.58$, it is considered advisable to accept that value. In other words, if human serum proteins which have undergone few, if any, physical changes in their preparation, are added (at pH 7.4) to a pure salt solution of specific conductivity about 136×10^4 at $25^\circ\text{C}.$, each additional gram of protein per 100 cc. will decrease the conductivity of the salt solution 1.58 per cent. This would produce a conductivity of about 120×10^4 for 7.5 per cent protein.

It should be noted that the variations in pH found in our solutions are insignificant because, at the pH of blood, changes in conductivity with change in hydrogen ion concentration are but slight.

A MICRO METHOD FOR THE DETERMINATION OF BASE IN BLOOD AND SERUM AND OTHER BIOLOGICAL MATERIALS.

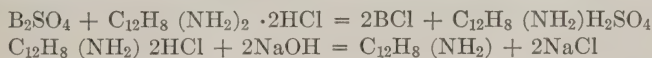
BY WILLIAM C. STADIE AND EFFIE C. ROSS.

*(From the John Herr Musser Department of Research Medicine, University of
Pennsylvania, Philadelphia.)*

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The use of benzidine hydrochloride to precipitate sulfates as insoluble benzidine sulfate was first proposed by Müller (1902) for the determination of free and bound sulfur in minerals. Subsequently Rosenheim and Drummond (1914) and Fiske (1921) used it for the determination of sulfates in urine. Later Fiske (1922) described a method for the determination of total base in urine, the bases after removal of phosphates being first converted to sulfates by ignition with sulfuric acid, precipitated as benzidine sulfate, the precipitate being then titrated with alkali.

The method here presented is one for the determination of base in blood, serum, urine, or any biological material. Similarly to Fiske's method the base is converted to sulfates by ignition with sulfuric acid and the equivalent sulfates are precipitated by benzidine hydrochloride. Unlike Fiske's method where the precipitate is directly titrated with alkali, base sulfates present are determined by titrating an aliquot of the filtrate from the benzidine sulfate. From this value and the titer of the benzidine hydrochloride reagent the concentration of base is readily calculated. The reactions involved are



The advantages of the indirect titration by difference are:

1. Elimination of quantitative transfer of a small precipitate.
2. Elimination of washing of precipitate with acetone.
3. Elimination of transfer back to titrating vessel of washed precipitate.

4. Elimination of hot titration of benzidine sulfate precipitate.
5. Elimination of a variable titration blank of filter paper.

The theoretical disadvantage of the abandonment of a direct titration for an indirect we have found to be non-existent. Careful scrutiny of sources of error likely to arise in the determination of base in blood and serum have convinced us that the method here described is reliable to about 1 per cent. In addition to indirect titration several other minor modifications are suggested which have reduced considerably the time and labor necessary for analysis.

The difficulties encountered in the presence of large amounts of phosphates have been clearly recognized by those employing the benzidine method in the analysis of biological material. On the basis of hundreds of analyses we are satisfied that no method so far proposed gives accurate results without prior removal of the interfering phosphates when they are present beyond a certain limit. For this purpose a simple technique is here described which together with the method of indirect titration greatly simplified total base determinations on urine, feces, gastric contents, etc.

Materials Needed.

Standardized $N/50$ NaOH.

Benzidine hydrochloride solution.

Benzidine.....	4 gm.
N HCl.....	45.0 cc.

Dissolve and make up to 250 cc. Filter if necessary. This solution is standardized by titrating 2.00 cc. against $N/50$ NaOH, using phenol red. It keeps indefinitely requiring occasional filtration. It is best to restandardize about once a week although as a rule the titer is unchanged over 2 to 4 weeks. Its equivalent concentration is about

Benzidine hydrochloride.....	175 m.-Eq. per liter.
Excess HCl.....	5 " " "
Total.....	180 m.-Eq. per liter.

10 cc. burettes graduated in 0.05 cc.

50 cc. silica beakers.

Method.

1.00 cc. of serum or an aliquot of a trichloroacetic acid filtrate of whole blood or cells (see below) corresponding to about 1 cc. of blood is placed in a 50 cc. silica beaker. Add 0.5 cc. of concentrated sulfuric acid and evaporate at low heat (about 90°C.) on a sand bath until practically all the water is driven off. Add 1 cc. of concentrated nitric acid and continue heating, raising the flame from time to time. Add 1 cc. of nitric acid occasionally to aid in the oxidation of organic matter. When the full heat of a triple burner has been reached and a clear white ash obtained, transfer to a triangle and heat 15 minutes at bright red heat, allowing the flame to play over the entire outside of the beaker. This as a rule suffices to drive off all excess of sulfuric acid. Let the beaker cool to room temperature, add 15.00 cc. of water. If preferred the ash may be dissolved and quantitatively transferred to a 20.00 cc. flask for precipitation. The ash readily dissolves with a little stirring. To be certain that no excess of sulfuric acid is present, add a drop of phenol red and titrate with N/50 NaOH. Rarely is more than 0.05 cc. required. This amount may be neglected but if more is necessary a correction must be made for it in the calculation. Add 2.00 cc. of standardized benzidine hydrochloride and filter through a small dry quantitative filter into a dry Erlenmeyer flask. Titrate 15.00 cc. of the filtrate with N/50 NaOH, adding more indicator if necessary.

*Calculation of Milli-Equivalents of Base per Liter of Blood
or Serum.*

For 1 cc. of blood or serum analyzed as above we have,

$$\begin{aligned} \text{m.-Eq. base per liter} &= (\text{titer of 2.00 cc. of benzidine HCl} - \frac{17}{15} \text{ titer of} \\ &\quad 15 \text{ cc. of filtrate}) = \text{cc. N/50 NaOH} \times 0.02 \times 1000 \end{aligned}$$

If the amounts given above are varied as occasion may direct we have for N/50 NaOH,

$$\begin{aligned} \text{m.-Eq. of base per liter or per kilo} &= 0.02 (\text{titer of } x \text{ cc. benzidine HCl} - \\ &\quad \frac{\text{total volume}}{\text{aliquot}} \times \text{titer aliquot}) \div \text{amount of material in liters or} \\ &\quad \text{kilos} \end{aligned}$$

If more than 0.05 cc. of N/50 alkali are required for the end-point of the ignited ash subtract from the titer of 2 cc. of benzidine HCl in above formula. Allowance, of course, is made in the "total volume" for the added volume which is increased by the added alkali.

Calculation of Base Bound to Phosphate.

As a rule the base in blood bound as phosphate is less than 2 milli-equivalents per liter and may for most purposes be neglected. Since the base as phosphate is not determined in this method, it must be separately determined when the phosphate concentration is a significant part of the total. The micro methods of Tisdall (1922) and Briggs (1924) are available. To the base as determined above add 1 milli-equivalent for each milli-equivalent of phosphorus. In the ignited ash, the phosphate is present as metaphosphate BPO_3 binding one equivalent of base per mol of phosphorus. If desired the phosphates may be removed by the method described below and the total base (including phosphate base) determined.

Preparation of Trichloroacetic Acid Filtrate of Whole Blood or Red Cells.

3.00 cc. of whole blood (or about 3 gm. accurately weighed of red cells) are placed in a 50 cc. volumetric flask. Add about 20 cc. of water and when hemolysis is complete add slowly, with vigorous shaking, 25 cc. of a 10 per cent trichloroacetic acid solution. Make up to volume and filter. Yield about 45 cc. 20 cc. of the filtrate are ashed as above.

Discussion of Sources of Error.

Amount of Material Needed.—The optimum concentration of base in the final total precipitating volume is about 8 to 10 milli-equivalents per liter. If, for example, the volume of the water in which the ash is dissolved plus the volume of benzidine HCl is 17 cc., then about 0.13 milli-equivalent of base is required. Making due allowance for loss on taking aliquots of the trichloroacetic acid filtrate in the case of blood and cells, the amounts of material necessary for a *single* analysis are

		Average base content.
	cc.	m.-Eq.
Whole blood.....	1.5	0.15
Serum.....	1.0	0.15
Cells.....	1.5	0.18

For duplicates, of course, double these quantities are necessary. Too close adherence to these amounts is not necessary as reliable results may be obtained with a final concentration of 5.0 to 25 milli-equivalents of base per liter in the final precipitating volume. They are merely offered as a guide to be varied with the kind of material and the quantity available for analysis.

Minimum and Maximum Amounts of Base Determinable.

Table I shows the results of analysis of K_2SO_4 . The total precipitating volume was 20 cc. Increasing amounts of base were taken giving the concentration indicated in Column 1.

TABLE I

Effect of Varying Final Concentration of Base in the Total Precipitating Volume. Ratio of Benzidine Hydrochloride to Base in M.-Eq. Constant = 1.6. Precipitating Volume = 20.0 cc.

No.	Final concentrations of base in precipitating volume.	Base taken.	Base found.*	Deviation.
	<i>m.-Eq. per l.</i>	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>per cent</i>
1	2.5	0.050	0.0480	-3.6
2	5.0	0.100	0.0990	-1.0
3	10.0	0.200	0.2018	+0.9
4	15.0	0.300	0.3027	+0.9
5	25.0	0.500	0.5020	+0.4
6	50.0	1.000	0.286	-1.4

* Mean of two concordant results.

Excellent results were obtained over a range of 5.0 to 25.0 milli-equivalents per liter in the final total precipitating volume. In other words, with a precipitating volume of 15 to 20 cc., from 0.08 to 0.5 milli-equivalent of base may be accurately determined or as little as 0.5 cc. of serum will suffice. Optimum results are obtained, however, when the final concentration of base is about 10 milli-equivalents per liter or 1 cc. of serum or blood to about 17 cc. of total precipitating volume.

Precipitation of Protein with Trichloroacetic Acid.

We have found that (1) the ratio of grams of trichloroacetic acid to grams of protein, (2) final concentration of trichloroacetic

acid, (3) and initial concentration of trichloroacetic acid are the essential factors which determine the yield of filtrate and freedom from turbidity. These conclusions in the main agree with the experience of Hiller and Van Slyke (1922) and are given only for the sake of completeness. In general a ratio of 5 gm. of trichloroacetic acid per gm. of protein, a final dilution of protein of 1 gm. to 100 cc., an initial concentration of 10 per cent, and a final concentration of 5 per cent of trichloroacetic acid are optimum conditions. Assuming an average protein content of 10 per cent for serum, 30 per cent for cells, and 20 per cent for whole blood, the optimum of conditions for protein precipitation per 1.0 cc. of material are

	Trichloroacetic acid.		Final volume.
	gm.	cc. of 10 per cent	cc.
Whole blood.....	2.0	10	20
Red blood cells.....	3.0	15	30
Serum.....	1.0	5	10

Under these conditions a yield of 90 per cent of water-clear filtrate is obtained.

Wet Ashing of Serum.—For serum alone we have found that precipitation of the protein is unnecessary. Direct ashing is easier and quicker as described under *Method*. The use of platinum evaporating dishes makes this technique difficult and uncertain as the material has a great tendency to creep over the sides of the dish resulting in serious loss of base. We strongly recommend the use of silica beakers which in our hands have given speed and accuracy to the method. Whole blood may be ashed directly also if preferred (see below).

Loss of Sulfate during Ignition.—We have repeatedly tested this possibility with both K_2SO_4 , Na_2SO_4 , and $NaCl$ and KCl plus sulfuric acid. We have been unable at any time to demonstrate any loss even after 1 hour's heating at bright red heat of small or large quantities of base. If the ash is heated too rapidly when still moist loss by mechanical crepitation may occur. This is easily avoided by heating to absolute dryness on a sand bath at about $100^\circ C$. before ignition. Table II shows the non-volatility of Na_2SO_4 and K_2SO_4 at bright red heat.

Use of Acetone as a Precipitating and Washing Medium.

The solubility of benzidine sulfate in water is sufficient to raise the possibility of incomplete precipitation of sulfate as benzidine sulfate and of excessive loss during washing. The first difficulty is non-existent as a consideration of Table III shows. Because benzidine sulfate is much less soluble in acetone,

TABLE II.

Non-Volatility of Na_2SO_4 and K_2SO_4 Heated to Bright Red Heat.

Time of heating.	Na_2SO_4	K_2SO_4	KCl and H_2SO_4
min.	mg.	mg.	mg.
0	64.3	68.3	127.5*
15	64.2		
30	64.2	67.1	127.7
60		67.2	
0	26.9		38.1*
30	27.0		38.2
60	26.9		

* KCl calculated as K_2SO_4 .

TABLE III.

Effect of Final Concentration of Acetone on the Estimation of Potassium Sulfate as Benzidine Sulfate.

Base taken 0.2034 m.-Eq.

No.	Acetone in total precipitating reaction mixture.	Base found.*	Deviation.
	vol. per cent	m.-Eq.	per cent
1	0	0.2044	+0.5
2	20	0.2030	-0.2
3	40	0.2034	0.0
4	60	0.2036	+0.1

* Mean of two concordant determinations.

Fiske (1922) introduced its use as a precipitating medium recommending a final concentration of 20 per cent. Table III shows, however, that the precipitation of sulfates by benzidine hydrochloride is quantitative in water alone. Addition of acetone is quite unnecessary. We have repeatedly tested filtrates for the presence of sulfates using BaCl_2 with negative results. In methods involving the washing of the benzidine sulfate precipi-

tate a loss undoubtedly results if water be used. The use of acetone and saturated benzidine sulfate solutions have been recommended. The former washes the precipitate effectively without loss. However, the titration of the excess benzidine as employed here renders this step unnecessary.

Ratio of Benzidine Hydrochloride to Base.

Throughout the literature of the benzidine method constant references are found to "adsorption" or "occlusion" of sulfates or benzidine hydrochloride by the precipitate of benzidine sulfate and frequent more or less rigid recommendations are made as to the conditions necessary for quantitative precipitation. A careful study of these factors has been made and within the

TABLE IV.

Effect of the Ratio of Benzidine Hydrochloride to Base.

Base taken (K_2SO_4) 0.200 m.-Eq.

No.	Ratio of benzidine HCl to base.	Base found.*	Deviation.
		<i>m.-Eq.</i>	<i>per cent</i>
1	1.0	0.1926	-3.7
2	1.4	0.1988	-0.6
3	1.6	0.2018	+0.9
4	3.0	0.1982	-0.9
5	4.0	0.1978	-1.1

* Average of triplicates.

requirements for base determinations in blood, serum, and urine, considerable latitude in conditions for quantitative precipitation is allowable.

The ratio of benzidine hydrochloride to base in milli-equivalents may be varied from 1.4 to 4.0 with results within the limit of error of the method (1 per cent). (Table IV.) When just sufficient benzidine hydrochloride is used the error due to the solubility of benzidine sulfate in water becomes apparent. Thus in No. 1 of Table IV the total volume was 20.0 cc. Not more than 1 per cent excess of benzidine hydrochloride was added. We may assume a saturated solution of benzidine sulfate. The solubility of benzidine sulfate being 0.7 milli-equivalent per liter, the loss due to the solubility of benzidine sulfate would be

0.02×0.7 or 0.014 milli-equivalent out of 0.200, *i.e.* 7 per cent. Actually the loss was 4 per cent. If an excess of benzidine hydrochloride (say 1 cc. of 0.2 equivalent) is used in 20 cc. we have, since the solubility product of benzidine sulfate in water is 0.7^2 milli-equivalent per liter,

$$(\text{SO}_4) = \frac{0.7^2}{0.2/0.02} = 0.049 \text{ milli-equivalent per liter}$$

as the amount of (SO_4) ion in solution. The loss of sulfate through solubility would be $\frac{0.049}{0.700}$, *i.e.* $\frac{1}{14}$ of the solubility in water or in an average base determination about 0.5 per cent. Both theoretically and practically therefore an excess of 100 per cent of benzidine insures practical insolubility of benzidine sulfate. Addition of acetone is unnecessary. It must be pointed out here also since the base is determined by the difference of two titers it is best to have the base titer about half of the total titer of the benzidine HCl used, *i.e.* to use about 100 per cent excess of the reagent. As is apparent from Table IV considerable latitude from this ratio is allowable without error.

Effect of Time after Precipitation and before Filtering.

This point was tested since it had been mentioned in the literature as a possible source of error. The results (Table V) are negative.

Precipitation at High Temperatures.

Müller (1902) asserts that high results are obtained due to occlusion by the precipitate of benzidine hydrochloride in the cold and may be avoided by hot precipitation. We have carefully tested this in a series of determinations at 75° and 100°C . The results, of which Table VI is one series, were consistent in showing that similar quantities of base are recovered in both hot and cold precipitation.

Effect of Acidity during Precipitation.

Since the titration of the excess benzidine hydrochloride used in this method requires the solution of base sulfates to be neutral

TABLE V.

Effect of Standing after Precipitation.

Base taken 0.2030 m.-Eq.

No.	Time standing after precipitation.	Base found.	Deviation.
	<i>min.</i>	<i>m.-Eq.</i>	<i>per cent.</i>
1	3	0.2028	-0.1
2	30	0.2024	-0.3
		0.2024	-0.3
3	60	0.2032	+0.1
		0.2040	+0.5
4	120	0.2040	+0.5
		0.2028	-0.1

TABLE VI.

Effect of Warming during Precipitation to 75°C.

Base taken 0.2030 m.-Eq.

No.	Base found.	Deviation.	Ratio of Benzidine HCl to base.
	<i>m.-Eq.</i>	<i>per cent</i>	
1	0.2028	-0.1	1.5
2	0.2032	+0.1	1.7
3	0.2022	-0.4	1.9
4	0.2044	+0.7	2.5
5	0.1996	-1.7	2.9

TABLE VII.

Effect of Increase of Acidity during Precipitation. Total Volume 25.00 Cc.

Base taken 0.2034 m.-Eq.

HCl added.	Total excess of HCl	Base found.	Deviation.
<i>m.-Eq. per 25 cc.</i>	<i>m.-Eq. per l.</i>	<i>m.-Eq.</i>	<i>per cent</i>
0	0*	0.2032	-0.1
0	5†	0.2032	-0.1
0.1	9	0.2020	-0.7
0.2	13	0.2036	+0.1
0.5	25	0.2044	+0.5

* Special reagents used with no excess of hydrochloric acid.

† Excess of HCl in benzidine HCl reagent.

before precipitation and moreover since conflicting statements exist (*vide*, Müller (1902), Raschig (1903), Rosenheim and Drummond (1914)) as to the requirements of the hydrogen ion concentration during precipitation a series of determinations was done with varying acidity. The results (Table VII) in water and with increasing acidity up to 0.025 N HCl are identical. The quantitative precipitation of benzdine sulfate is independent of pH over a wide range approximately from 7.0 to 1.6.

Comparison of Base Determination of Serum by Direct Wet Ashing and by Trichloroacetic Acid Precipitation of Protein and Ashing by Filtrate.

When serum alone is analyzed the trichloroacetic acid precipitation of proteins is quite unnecessary and introduces needless and tedious steps into the analysis. No appreciable metallic base is bound "organically" with serum proteins so that direct wet ashing gives the total base $BP + BHCO_3 + BCl$ present in serum. These base moieties together with the base bound as phosphate are the ones usually desired. For whole blood or red cells the presence of iron organically bound with the hemoglobin offers no bar to the direct ashing of blood since the iron present is completely converted to Fe_2O_3 on strong ignition. While the direct ashing of serum saves time and is strongly recommended the direct ashing of whole blood or cells is a matter of choice. In practice we prefer the protein precipitation and ashing of the filtrate as we have found that the ashing of whole blood is slower than serum.

The assumption that in this precipitation no inorganic base is carried down with the precipitate is a natural one but apparently has never, so far as the literature is concerned, been tested.

Table VIII shows a comparison of the analysis of serum and whole blood with and without added base directly ashed and ashed after precipitation of the protein with trichloroacetic acid. The results show agreement to within 1.0 per cent both for the base present in the serum and for the recovery of added base. For whole blood the difference is 1.6 per cent. The assumption seems warranted that for blood, as well as for serum, analyses of trichloroacetic acid filtrates represent the total inorganic base (less phosphate base) present.

TABLE VIII.

Analysis of Base in Serum and Whole Blood (1) by Wet Ashing with Sulfuric Acid and (2) by Trichloroacetic Acid Precipitation of Protein.

		Wet ashing. Base found.	Trichloroacetic acid precipitation of protein. Base found.
		<i>m.-Eq. per l.</i>	<i>m.-Eq. per l.</i>
I. Serum.	1	165.5	165.9
	2	163.0	164.8
	3	162.5	
	Base found, mean.....	163.7 \pm 1.2	165.4 \pm 0.5
	“ added KCl.....	50.5	40.4
	1	212.3	205.7
	2	212.0	205.8
	“ found, mean.....	212.1 \pm 0.1	205.8 \pm 0.0
	Total base taken, mean.....	214.2 \pm 1.2	205.8 \pm 0.5
	Deviation.....	0.5 per cent	0.2 per cent.
II. Whole blood.		136.8	137.5
		135.6	139.5
	Base found, mean.....	136.2 \pm 0.6	138.5 \pm 1.0
	Deviation.....	-1.6 per cent.	

TABLE IX.

Complete Recovery of Calcium Sulfate with Total Base.

Base taken (as K_2SO_4) 0.200 m.-Eq.

No.	Calcium sulfate taken.	Total base taken.	Base found.*	Deviation from base taken.
	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>per cent</i>
1	0	0.2000	0.2006	+0.3
2	0.0054	0.2054	0.2068	+0.7
3	0.0158	0.2158	0.2146	-0.6
4	0.0422	0.2422	0.2412	-0.5

* Mean of concordant duplicates.

Recovery of Calcium.

The solubility in water of calcium sulfate is about 26 milliequivalents per liter. In the presence of potassium or sodium sulfate this is reduced but for the conditions under which the

ash from a single determination is dissolved the solubility is still 20 milli-equivalents per liter. Table IX shows that this amount of calcium sulfate together with 100 milli-equivalents of potassium, a ratio far exceeding that in body fluids, is completely recovered in the total base analysis.

Effect of Phosphate on the Determination of Base as Benzidine Sulfate.

The presence of phosphates in blood and serum is the most prolific source of error in the benzidine sulfate method. We have convinced ourselves by a close examination of the factors involved that in the method as here described the presence of 10 milli-equivalents of phosphorus per 100 to 150 milli-equivalents

TABLE X.

Time Required at Bright Red Heat for Complete Conversion of KH_2PO_4 to Monobasic Orthophosphate (KH_2PO_4) to Metaphosphate (KPO_3).

No.	Time of heating to red heat.	KPO_3 calculated.*	KPO_3 found.
	<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>
2	1	110.2	110.3
3	$\frac{1}{2}$	17.3	17.5

* Calculated as 86.7 per cent of KH_2PO_4 taken.

of base does not increase the error of the method beyond the 1 to 2 per cent variation. Since for normal blood, serum, and cells the average phosphate content is about 2 milli-equivalents per 130 milli-equivalents of base of 1.5 per 100 a seven-fold increase in phosphate is allowable without increased error. Bloods containing more phosphates than this are extremely rare. When encountered the possibility of error in the base by the benzidine method must be borne in mind.

Effect of Metaphosphate.

When an acid phosphate is heated it is converted into metaphosphate (BPO_3). The conversion, if large quantities are heated, requires many hours but for small quantities it is complete in $\frac{1}{4}$ to $\frac{1}{2}$ hour as Table X shows. We have determined the solubility of potassium metaphosphate so prepared and found

it to be 0.1 milli-equivalent per liter. In the presence of other salts the solubility is increased. The following table shows the solubilities of potassium metaphosphate at 20°C. in sulfates of blood cations.

	Solubility of KPO_3
	<i>m-Eq. per l.</i>
Water.....	0.1
0.1 eq. K_2SO_4	0.1
0.1 eq. Na_2SO_4	17.0
Saturated $CaSO_4$	23.0
0.0100 eq. K_2SO_4 } *	2.0
0.0005 eq. $CaSO_4$ }	

* This is the approximate composition of a solution of ash from 1 cc. of cells in 15 cc. of H_2O .

The solubility of metaphosphate in the solution of the blood ash sulfates would be difficult to predict, but its relative insolubility would lead one to expect that only a part of the metaphosphate would go into solution. Such is the case as is shown in Table XI.

2 cc. of the solution analyzed gave an ash containing 0.200 milli-equivalent of K_2SO_4 , 0.005 $CaSO_4$, and 0.0132 P. The ash was dissolved in 10 cc. of water stirred and filtered. The filtrate was analyzed for phosphorus by the Briggs method. It is apparent that by adequate heating complete conversion of phosphate to metaphosphate is accomplished and as a result there was a removal of 50 to 60 per cent of interfering phosphate on account of its insolubility as a metaphosphate. Tables XII and XIII show that, as a result of this partial removal of phosphates, the upper safe limit of phosphate is increased to about 10 milli-equivalents per 100 milli-equivalents of base.

A brief examination of Table XIII shows what we have consistently found, *viz.* when the phosphates are low (0.005 and 0.010 milli-equivalent to about 0.150 milli-equivalent of base) theoretical results are obtained. When somewhat higher (0.02 milli-equivalent) theoretical recoveries are the rule, except when the cation is all sodium or all potassium in which case the results are higher. With higher concentrations of phosphate the results are always high.

We have made a diligent effort to satisfy ourselves that the presence of phosphates does not interfere with the determination

TABLE XI.

Conversion at Red Heat and Partial Removal of Phosphates as Sparingly Soluble Metaphosphates.

Solution analyzed:

KCl 100.0 m.-Eq. per l.

CaCl₂ 2.5 " " "

2.00 cc. plus 0.0132 m.-Eq. P as KH₂PO₄ taken.

Base found by authors' method 102.5 m.-Eq. per l.

Condition.	Phosphorus found in ash filtrate.	Phosphate removed as metaphosphate.
	<i>m.-Eq. × 1000</i>	<i>per cent</i>
Insufficient heating (H ₂ SO ₄ acid left)...	11.6	12
Ignition 1 hr. Digestion with 10 cc. H ₂ O and 2 drops of concentrated HCl at 100°C.....	13.1	2
Ignition 1 hr. Ash dissolved in 0.004 N HCl.....	5.5	59
Ignited 20 min. Ash dissolved in H ₂ O..	4.8	64
Ignited 1 hr. Ash dissolved in H ₂ O.....	5.7	57

TABLE XII.

Determination of Potassium and Calcium Chlorides in the Presence of Varying Phosphate.

Base..... KCl 0.200 m.-Eq.

CaCl₂ 0.005 "

Total less base

as phosphate.... 0.205 m.-Eq.

No.	KH ₂ PO ₄	Base found.*	Deviation from total base less base as phosphate.
	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>per cent</i>
1	0.000	0.2048	-0.1
2	0.0025	0.2070	+1.0
3	0.005	0.2056	+0.3
4	0.015	0.2062	+0.6
5	0.025	0.2006	-1.8

* Mean of concordant triplicates.

of base when the precipitation was carried on at an acidity of pH 3 to 4 (acid to Congo red) as affirmed by Rosenheim and

Drummond (1914) and Fiske (1921). This method necessitates, of course, a direct titration of the precipitate. As a result of some hundreds of analyses we are prepared to state very definitely that high phosphate (over 0.020 to 0.150 milli-equivalent of base in about 25 cc. of precipitating volume) invariably gives results high by 2 to 20 per cent. Below this figure high results (3 to 6 per cent) may be and often are obtained. When by reason of the necessity for ashing, the material analyzed has been ignited with a resulting partial or complete conversion of orthophosphate to metaphosphate the results obtained are much

TABLE XIII.

Determination of Base in the Presence of Varying Phosphate and Varying Proportions of Sodium and Potassium.

PO ₄	Na	K	Total base less phosphate.		Deviation.
			Calculated.	Observed.	
<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>per cent</i>
0.005	0.160	0.000	0.155	0.1566	+1.0
0.010	0.170	0.000	0.160	0.1662	+4.0
0.010	0.120	0.050	0.160	0.1594	+0.3
0.010	0.000	0.215	0.205	0.206	+0.6
0.020	0.240	0.000	0.220	0.238	+8.2
0.020	0.140	0.050	0.170	0.1685	-0.9
0.020	0.090	0.100	0.170	0.1711	+0.7
0.020	0.000	0.170	0.150	0.1579	+5.2
0.050	0.150	0.050	0.150	0.1619	+8.0
0.050	0.100	0.100	0.150	0.1549	+3.3
0.050	0.200	0.000	0.150	0.1514	+0.9
0.050	0.000	0.200	0.150	0.164	+9.8

more irregular and as a rule run 4 to 6 per cent too high even when the concentration of the phosphates is 5 to 10 milli-equivalents per liter, *i.e.* within the limits of pathological bloods. It would be useless to publish a multiplicity of data bearing on this question so that only Table XIV is offered which represents the best series we have been able to obtain with acid precipitation and direct titration of the precipitate.

The general conclusions reached from a consideration of these factors are:

1. For practically all sera and bloods the total base less the

base bound as phosphate may be determined by the method of indirect titration. The phosphate concentration must be less than 10 milli-equivalents per liter.

2. For urine, feces, gastric contents, etc. with high phosphate the total base may be determined only after removal of phosphate by the method outlined below.

3. The method of determination of the total base minus the phosphate base by precipitation in acid solution (Fiske technique) with direct titration of the precipitate is unreliable with high phosphate and offers no guaranty of greater accuracy with low phosphates than the method described here.

TABLE XIV.

Determination of Base in Presence of Varying Phosphate. Precipitation at pH 3.0 (Acid to Congo Red) without Prior Ignition. Direct Titration of Precipitate.

Na_2HPO_4	Na_2SO_4	Base observed.*	Deviation.
	<i>m.-Eq.</i>	<i>m.-Eq.</i>	<i>per cent</i>
0.010	0.152	0.1535	+1.0
0.020	0.152	0.1547	+1.8
0.050	0.152	0.1624	+6.8
0.050†	0.150	0.1592	+6.1

* Mean of 5 concordant determinations.

† Acid to methyl violet (pH about 1.5).

Removal of Phosphate and Recovery of Total Base.

When the ratio in equivalents of phosphate to base exceeds 10 to 150, biological material cannot be analyzed with certainty by the benzidine method. This holds true whether the precipitation is done in neutral solution or at a pH just acid to Congo red. Blood specimens even in cases of marked phosphate retention (as in nephritis) will rarely offer any difficulty on this score but urine and other material having a high phosphate content relative to base must first have the phosphate removed. The total base may then be determined with ease and accuracy by the method of indirect titration.

We have tested carefully a simple and expeditious method of precipitating all the phosphate as ferric phosphate and the excess of iron as ferric hydroxide. The filtrate containing all

the base is ignited with sulfuric acid and the sulfates determined as for blood. In principle this was proposed by Fiske (1922) who, however, employed ammonium acetate as a precipitating agent necessitating a hot filtration after boiling. In practice we found this tedious and moreover uncertain, as frequently a large part of the phosphate was redissolved as a result of the inevitable cooling which recurred during filtration. Indeed Fiske warns that the filtration should not take longer than 2 minutes. This we found difficult to accomplish with a precipitate of the character of ferric hydroxide. Using dilute NH_4OH it is possible to precipitate completely in the cold all of the phosphate together with the excess of iron. Sufficient NH_4OH is

TABLE XV.
Removal of Phosphates as Ferric Phosphate. Volume 10 Cc.

Approximate pH.	Na_2HPO_4	Filtrate.	
		PO_4^{---}	Fe^{+++}
	<i>mM</i>		
6.0	0.010*	0	0
8.0	0.010	0	0
8.0 + 1 cc. 0.1 N NH_4OH	0.010	+	0
8.0 + 2 cc. 0.1 N NH_4OH .	0.010	++	0
8.0 + 5 cc. 0.1 N NH_4OH .	0.010	+++	0

* Identical results are obtained with 0.020 and 0.050 mM Na_2HPO_4 .

used to produce a full red with phenol red as indicator. An excess of NH_4OH inevitably redissolved some of the phosphate yet there is a sufficient margin of safety so that no particularly delicate adjustment of the reaction is necessary. We have found that complete removal of the phosphates is accomplished between a reaction of pH 6 to 8.

Table XV indicates qualitatively the removal of phosphates under the conditions stated and Table XVI gives the quantitative data showing complete recovery of total base after removal of varying quantities of phosphates.

Occasionally traces of iron appear in the filtrate but on ignition ferric sulfate is completely converted over into Fe_2O_3 . This in no way interferes with base quantitation as is clearly indicated in

Table XVII which shows that even a large amount of Fe_2O_3 is not a source of error.

Technique for the Removal of Phosphate.—The material (urine, tissue, gastric contents, feces, etc.) in an amount sufficient to

TABLE XVI.

Removal of Phosphate and Recovery of Total Base.

Constant: Na_2SO_4 0.1500 m.-Eq.

Na_2HPO_4 taken.	Total base.		Deviation.
	Calculated.	Observed.	
mm	m.-Eq.	m.-Eq.	per cent
0.005	0.1600	0.1621	+1.3
0.005	0.1600	0.1610	+0.6
0.005	0.1600	0.1605	+0.3
0.010	0.1700	0.1657	-2.5
0.010	0.1700	0.1693	-0.4
0.010	0.1700	0.1694	-0.4
0.020	0.1900	0.1945	+2.3
0.020	0.1900	0.1876	-1.2
0.020	0.1900	0.1880	-1.0
0.050	0.2500	0.2504	+0.2
0.050	0.2500	0.2450	-2.0
0.050	0.2500	0.2504	+0.2
0.050	0.2500	0.2500	+0.0
0.050	0.2500	0.2540	+0.2

TABLE XVII.

Non-Interference of Fe_2O_3 Obtained by the Ignition of Ferric Sulfate on the Precipitation of Sulfates by Benzidine Hydrochloride.

Constants: Na_2SO_4 0.152 m.-Eq.

$\text{Fe}_2(\text{SO}_4)_3$ 0.100 "

Base observed.	Deviation from calculated.
m.-Eq.	per cent
0.1520	0.0
0.1526	+0.4
0.1520	0.0

give about 0.100 to 0.200 milli-equivalent of base is digested in a silica beaker with sulfuric and nitric acids until the organic matter is oxidized. Do not ignite to complete dryness at any time. Transfer to a 25 cc. volumetric flask using about

15 cc. of H_2O , add a few drops of phenol red; neutralize with 4 N NH_4OH (1:4 concentrated NH_4OH). Render just acid with a drop or two of normal H_2SO_4 . Add 0.1 N ferric ammonium sulfate (0.033 M) using about 6 equivalents of iron for every equivalent of phosphate. In practice 1 cc. of 0.1 N ferric alum will completely precipitate up to 0.020 milli-equivalent of phosphate in the sample. Add 0.1 N NH_4OH just to a full red to the indicator and filter. Transfer 20.00 cc. of the filtrate to a silica beaker or platinum dish, add a drop of concentrated H_2SO_4 , evaporate, and ignite, continuing the analysis as outlined under method for blood. In the calculation, of course, due allowance is made for the aliquot taken.

SUMMARY.

A micro method for the determination of total base in whole blood, cells, serum, and other biological materials is described. The bases are converted to sulfate, precipitated by benzidine hydrochloride, and the benzidine sulfate determined by titration of the excess of benzidine hydrochloride.

The sources of errors are discussed. In the absence of excessive quantities of phosphate the method is reliable to 1.0 per cent.

Biological materials containing high phosphates may be analyzed by the method after removal of the phosphates, for which a simple technique is described.

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CHANGES IN THE COMPOSITION OF THE URINE AFTER MUSCULAR EXERCISE.*

By D. WRIGHT WILSON, W. L. LONG, H. C. THOMPSON, AND
SYLVA THURLOW.

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)

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The effect of strenuous muscular exercise on the body has been studied extensively. The responses of many of the mechanisms which take part in attempting to furnish oxygen and fuel and to remove carbon dioxide and other waste products have been analyzed in considerable detail. Studies on the respiratory exchange, the blood, and the urine have contributed much toward furnishing us with a detailed knowledge concerning the extent to which the mechanisms for maintaining neutrality in the organism are brought into play.

The work of Barr and his collaborators (1923, *a*, *b*, and *c*) has strikingly demonstrated the relatively great variations in the reaction of the blood brought about by short periods of strenuous exercise. One would expect that such large variations in the blood would be accompanied by a response by the kidney resulting in similarly great changes in the composition of the urine. The data in the literature which permit such comparisons are, however, meager and sometimes inconclusive and contradictory. In most of the older work, long periods of exercise and long periods of urine collection were employed. We believe that our studies of short periods of strenuous exercise and short periods of urine collection have yielded data of greater value for showing the immediate effects of the exercise and attempts at compensation on the part of the body.

* A preliminary announcement was made in the Proceedings of the Society for Experimental Biology and Medicine (Wilson, D. W., Long, W. L., Thompson, H. C., and Thurlow, S., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 425).

EXPERIMENTAL.

Urine was collected from normal men at frequent intervals before and after short periods of strenuous exercise. In most of the experiments the urine was collected every 10 minutes. No difficulty was experienced in voiding at such frequent intervals and the data indicate that such short periods of collection are quite suitable for studies such as these and ought to be useful in other connections. It is impossible to state how much overlapping of periods occurred due to the incomplete removal of all urine from the urinary system but it obviously did not interfere with the main purpose of the experiment. Low urine volumes after exercise associated with an increased excretion of certain constituents, together with a moderately constant output of creatinine, demonstrate that the observed variations are not due to errors of collection. Our controls demonstrate that our experiments were of sufficiently brief duration not to be influenced appreciably by the diurnal variations which have been shown to occur by Simpson (1924) and others.

The urines were voided directly into measuring cylinders containing toluene to diminish the loss of CO_2 and the consequent change in reaction. After recording the amount collected, the sample was diluted to constant volume with boiled neutral distilled water and stirred carefully under toluene. The reaction was estimated at once colorimetrically. The acidity was determined by titrating with phenolphthalein. In the early experiments, nearly all of a 5 or 10 minute specimen of urine was used, potassium oxalate added, and the titration made without dilution. The titration varied from 1 to 5 cc. In the later experiments, one-fifth or one-tenth of a 10 minute specimen was used, diluted with neutral distilled water until there was no tendency for the calcium phosphate to precipitate (Fiske, 1921), and the titration carried out with a micro burette. The titrations varied from 0.2 to 1.0 cc. Ammonia was determined by the micro method of Folin and Bell (1917), creatinine by Folin's micro method, total nitrogen by Kjeldahl, and phosphates by Briggs' (1922) modification of Bell and Doisy's (1920) method. Chlorides¹ were determined by a slight modification of Whitehorn's (1920-

¹ Miss Cecilia Riegel carried out the chloride analyses.

21) method, using 1 cc. of urine. The quantity of urine passed in 10 minutes is sufficient for suitable analyses by these methods. Determinations for acidity, total nitrogen, and chlorides were made in duplicate. Colorimetric determinations for pH, ammonia, phosphate, and creatinine were not regularly made in duplicate though many duplicates were run.

All of the experiments were carried out in the early afternoon on days when no lunch had been taken and only the ordinary activity of a student had been undergone. After a suitable fore period, the urine was voided and exercise was begun at once by running up and down one flight of stairs as rapidly as possible, swinging the arms vigorously. After the exercise the subject rested in a chair for 10 or 15 minutes and then usually resumed some laboratory work involving but little activity.

In Tables I to XII are recorded the data of a number of experiments performed on two subjects. All data involving quantity output are expressed in terms of excretion per minute.

In the early experiments (Tables I, II, III, and IV) running up and down one flight of stairs was undertaken at a moderate rate of speed. The exercise extended over periods of from 1 to 3 minutes in which time from two to seven round trips were made. This amount of exercise was strenuous and led to hyperpnea, but later the rate of running was about doubled. The urine was collected in periods of 5 or 10 minutes and in one case 2 minute periods were made use of immediately after the exercise.

The data from these experiments demonstrate that after short periods of strenuous exercise there is a sharp rise in the excretion of acids and ammonia and a diminution in pH and urine volume. An attempt was made in these and in subsequent experiments, frequently without success, to eliminate the change in urine volume after exercise by drinking water. The rise in the acidity factors reached a maximum in about 10 to 15 minutes after exercise and thereafter diminished, returning to normal in 30 to 45 minutes.

An examination of the results obtained in the 2 and 5 minute collections during and after exercise shows that there is a delay of several minutes before the changes described above begin to appear in the voided urine. During this interval the pH changes little or not at all, but the titratable acidity falls. The urine volume

TABLE I.

May 10, 1923. Strenuous exercise 3 min., 4.18-4.21 p.m. 7 round trips. Subject W. Ingested 200 cc. water at 3.50 p.m. Figures represent excretion per minute.

Time.*	Period.	Volume.	pH	Acid.
<i>p. m.</i>	<i>min.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>
4.08	5	0.9	6.2	0.34
4.13	5	0.7		0.31
4.18	5	1.2		0.34
4.23	5	0.8	5.2	0.20
4.28	5	1.0		0.60
4.33	5	2.0		0.90
4.38	5	1.6		0.73
4.43	5	1.0		0.60
4.48	5	0.8		0.35
4.53	5	0.8		0.42
4.58	5	0.8		0.43
5.03	5	0.9		0.46
5.08	5	0.9		0.30
5.13	5	1.0		0.37

* Column 1 records the end of the period of collection in all tables.

TABLE II.

May 12, 1923. Strenuous exercise 2 min., 12.07-12.09 p.m. 3 round trips. Subject W. Figures represent excretion per minute.

Time.	Period.	Volume.	pH	Acid.
<i>a. m.</i>	<i>min.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>
11.52	5	1.3	6.6	0.21
11.57	5	1.3	6.9	0.16
<i>p. m.</i>				
12.02	5	1.3	6.6	0.20
12.07	5	1.2	6.6	0.17
12.12	5	0.7	6.3	0.13
12.17	5	0.9	6.0	0.49
12.22	5	0.8	5.0	0.49
12.27	5	0.5		
12.32	5	0.4	5.0	0.34
12.37	5	0.4	5.2	0.29
12.42	5	0.8	5.4	0.44

tends to be lower than that of the periods preceding or following. It is difficult to be certain in these very short periods of collection

TABLE III.

May 17, 1923. Strenuous exercise 1 min., 12.59–1.00 p.m. 3 round trips. Subject W. Ingested 200 cc. water at 12 noon, 50 cc. at 1.02, and 50 cc. at 1.40 p.m. Figures represent excretion per minute.

Time.	Period.	Volume.	pH	Acid.	NH ₃
<i>p. m.</i>	<i>min.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>
12.38	10	4.5	6.5	0.247	0.133
12.48	10	3.4	6.5	0.207	0.108
12.58	10	4.0	6.6	0.230	0.141
1.03	5	0.7	6.6	0.037	
1.13	10	2.6	5.5	0.550	0.407
1.23	10	1.8	5.5	0.403	0.315
1.33	10	1.9	5.7	0.316	0.243
1.43	10	0.7	5.7	0.204	0.154
1.53	10	0.6	5.7	0.262	0.168
2.03	10	0.9	5.7	0.280	0.208

TABLE IV.

May 23, 1923. Strenuous exercise 1 min., 11.00–11.01 a.m. 2 round trips. Subject W. Ingested 100 cc. water at beginning of experiment. Figures represent excretion per minute.

Time.	Period.	Volume.	pH	Acid.	NH ₃
<i>a. m.</i>	<i>min.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>
10.40	10	1.0	7.0	0.096	0.078
10.50	10	1.1	7.2	0.088	0.069
11.00	10	2.1	7.0	0.160	0.106
11.02	2	2.1	7.0		
11.04	2	1.5	6.3		
11.14	10	4.2	5.5	0.479	0.279
11.24	10	3.6	6.0	0.213	0.121
11.34	10	5.6	6.8	0.242	0.102
11.44	10	2.3	6.9	0.152	0.087
11.54	10	1.2	7.1	0.128	0.066
12.04	10	0.9	7.2	0.112	

that the low acidities and urine volumes are real on account of the uncertainty of complete emptying of the bladder. Some difficulty

was experienced in voiding during the first few minutes after exercise due to fatigue. It is thought, however, that the observations are reasonably correct and may perhaps be explained by a sudden shutting down of activity on the part of the kidney due to diminished blood flow through it.

The pH of such urine specimens must also be influenced by the contamination of the specimen by the portions of fluid still remaining in the urinary tract. The fact that Barr and his collaborators have shown that there is an immediate and rapid change in the reaction of the blood in strenuous exercise suggests that the 2 or 3 minute delay in observing any change in pH of the urine represents the period of time necessary to flush out the urinary tract with the diminished flow of urine.

A more extended series of analyses on each urine specimen was planned and in order to have sufficient material for analysis it was decided to make collections in 10 minute periods. Control experiments were carried out in which collections were made under conditions similar to those upon which the exercise was superimposed. Each experiment was started early in the afternoon, on a day when no lunch had been eaten, and the accustomed activities had occurred in the morning (except in one control experiment). During the experiments moderate activity in the laboratory was continued. Attempts were made to maintain the urine volumes constant by drinking water during the experiments.

An examination of the data obtained from four control experiments on two individuals shows that during the period of 2 hours or less used for the experiment no great variations occurred although both chlorides and total nitrogen diminished at a rather uniform rate. In a single experiment the inorganic phosphate excretion was fairly constant.

While considering normal values it is of interest to examine the magnitude of certain of the data obtained in the experiments on the different subjects, making use of the normal values included in the fore periods of the exercise experiments also. The urines of Subject W. were almost always more alkaline than those of Subject L. The range was: Subject W., pH 6.2 to 7.4; Subject L., pH 5.4 to 6.2. The single exception occurred in a control experiment on Subject W., undertaken after an experiment involving strenuous exercise 3 hours previously. The urines with high pH

were found to contain the minimum amounts of titratable acids and ammonia. In the one experiment which permitted the calculation, the ammonia nitrogen was found to be only 1 per cent of the total nitrogen at a time when the pH ranged between 6.6 to 6.9. The analyses were carefully checked so that we are confident that the results are correct. Values of the percentage of ammonia

TABLE V.

May 21, 1923. Control. Subject W. Ingested 100 cc. water at 11.55 a.m. Figures represent excretion per minute.

Time.	Period.	Volume.	pH	Acid.	NH ₃	Creatinine.
<i>p. m.</i>	<i>min.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>
12.10	10	0.95	7.4	0.091	0.055	1.05
12.20	10	2.15	7.1	0.133	0.057	1.15
12.30	10	3.40	6.9	0.168	0.089	1.03
12.40	10	4.25	6.9	0.212	0.091	1.28
12.50	10	3.15	6.9	0.195	0.082	1.08
1.00	10	2.35	7.0	0.181	0.080	1.05

TABLE VI.

May 30, 1924. Control. Subject W. (After strenuous exercise in morning.) Figures represent excretion per minute.

Time.	Volume.	pH	Acid.	NH ₃	Total N	$\frac{\text{NH}_3\text{-N}}{\text{Total N}}$	Cl
<i>p. m.</i>	<i>cc.</i>		<i>cc. 0.1N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>	<i>per cent</i>	<i>cc. 0.1 N</i>
2.20	3.90	5.3	0.323	0.252	9.56	3.69	1.48
2.30	4.55	5.3	0.294	0.237	10.10	3.29	1.23
2.40	5.90	5.4	0.346	0.254	10.95	3.25	0.93
2.50	6.80	5.6	0.384	0.231	8.48	3.82	0.83
3.00	3.60	5.2	0.323	0.206	7.74	3.72	0.88
3.10	2.35	5.1	0.364	0.242	8.10	4.19	0.70
3.20	1.60	5.1	0.399	0.241			0.85
3.30	1.50	5.0	0.399	0.251	7.68	4.56	0.75
3.40	2.10	5.3	0.388	0.249	7.71	4.34	0.73
3.50	1.30	5.1	0.413	0.222	7.71	4.02	0.73

nitrogen as low as these may be inferred from some of Simpson's data which include figures for ammonia and urea. It is apparent that, for urine specimens collected during short periods, the normal values for the proportion of ammonia nitrogen to total nitrogen will vary over a much wider range than those found to hold for 24 hour specimens.

The variations caused by short periods of strenuous exercise were clean-cut and striking. There was a diminution in the

TABLE VII.

Mar. 24, 1924. Control. Subject L. Ingested water every 20 min., 200 cc. from 1.30, 100 cc. from 3.05 p.m. Figures represent excretion per minute.

Time.	Volume.	pH	Acid.	NH ₃	Creatinine.
<i>p. m.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>
2.35	7.9	5.8	0.649	0.239	1.19
2.45	8.5	5.8	0.675	0.287	1.14
2.55	9.8	5.8	0.530	0.279	1.29
3.05	9.0	5.8	0.526	0.387	1.12
3.15	7.9	5.8	0.490	0.327	0.96
3.25	8.3	5.8	0.439	0.263	1.16
3.35	7.2	5.8	0.477	0.364	1.03
3.45	7.5	5.8	0.447	0.269	0.96
3.55	7.6	5.8	0.435	0.302	1.14

TABLE VIII.

May 19, 1924. Control. Subject L. Water every 15 min., 100 cc. at 1.00 p.m. and thereafter until 2.00 p.m., then 50 cc. thereafter. Figures represent excretion per minute.

Time.	Volume.	pH	Acid.	NH ₃	Total N.	PO ₄	NH ₃ -N Total N	Cl
<i>p. m.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>	<i>cc. 0.1 M</i>	<i>per cent</i>	<i>cc. 0.1 N</i>
2.00	4.20	6.2	0.293	0.211	10.05	0.123	2.95	1.31
2.10	5.77	6.2	0.285	0.223	8.06	0.157	3.87	1.00
2.20	5.80	6.2	0.286	0.207	8.24	0.154	3.52	0.83
2.30	4.30	6.2	0.235	0.203	7.74	0.134	3.67	0.66
2.40	3.05	6.1	0.229	0.213	7.59	0.136	3.94	0.80
2.50	2.25	5.8	0.188	0.181	6.47	0.129	3.93	0.61
3.00	1.90	5.8	0.258	0.213	7.38	0.151	4.04	0.63
3.10	2.17	5.8	0.258	0.184	7.87	0.144	3.28	0.54
3.20	2.13	5.8	0.258	0.203	7.54	0.136	3.76	0.34
3.30	2.23	5.8	0.252	0.194	7.32	0.138	3.71	0.37
3.40	1.42	5.6	0.258	0.188	6.13		4.29	0.37
3.50	0.90	5.4	0.270	0.181	6.02	0.112	4.21	0.32

volume of urine excreted which could not be overcome by drinking even relatively large amounts of water. In Experiment 12 (Table XII) 200 cc. of water were ingested every 30 minutes for a

TABLE IX.

Apr. 15, 1924. Strenuous exercise 2 min., 2.05-2.07 p.m. Subject W. Ingested 100 cc. water at 1.50 and at 2.03 p.m. Figures represent excretion per minute.

Time.	Volume.	pH	Acid.	NH ₃	Total N.	$\frac{\text{NH}_3\text{-N}}{\text{Total N}}$	PO ₄	Cl	Creatinine.
<i>p. m.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>	<i>per cent</i>	<i>cc. 0.1 M</i>	<i>cc. 0.1 N</i>	<i>mg.</i>
1.44	3.45	6.9	0.530	0.072	9.68	1.04	0.293	1.52	1.19
1.54	5.05	6.8	0.402	0.068	9.66	1.00	0.245	1.43	1.00
2.04	4.90	6.6	0.398	0.061	8.37	1.03	0.248	1.22	1.03
2.14	3.95	6.0	0.530	0.233	7.46	4.33	0.328	0.43	1.06
2.24	3.50	5.3	0.795	0.318	7.48	5.95	0.467	0.22	1.10
2.34	1.10	5.2	0.596	0.260	6.56	4.97	0.336	0.45	1.03
2.44	1.25	5.2	0.635	0.176	7.86	3.13	0.304	0.80	1.08
2.54	2.90	5.6	0.424	0.116	10.30	1.57	0.235	1.04	1.03
3.04	3.30	6.1	0.371	0.086	8.16	1.48	0.177	1.07	0.99
3.14	4.90	6.4	0.331	0.114	9.86	1.61	0.199	1.54	1.07
3.24	3.90	6.4	0.291	0.099	8.40	1.64	0.152	1.21	
3.34	2.60	6.4	0.331	0.074	8.38	1.24	0.147	1.43	1.06

TABLE X.

Apr. 7, 1924. Strenuous exercise 2 min., 3.16-3.18 p.m. Subject L. Water ingested every 15 min., 25 cc. until 3.00 p.m. then 40 cc. Figures represent excretion per minute.

Time.	Volume.	pH	Acid.	NH ₃	Total N.	$\frac{\text{NH}_3\text{-N}}{\text{Total N}}$	PO ₄	Cl	Creatinine.
<i>p. m.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>	<i>per cent</i>	<i>cc. 0.1 M</i>	<i>cc. 0.1 N</i>	<i>mg.</i>
2.45	2.40	5.4	0.397	0.249	10.26	3.48	0.146	1.21	1.05
2.55	1.95	5.4	0.478	0.294	9.43	4.38	0.161	1.23	1.08
3.05	1.75	5.4	0.494	0.315	9.61	4.59	0.159	1.13	1.12
3.15	2.20	5.4	0.476	0.317	10.20	4.35	0.183	1.19	1.14
3.25	1.48	5.2	0.782	0.564	7.12	11.08	0.239	0.52	1.11
3.35	1.40	5.0	0.874	0.658	7.34	12.55	0.382	0.23	1.21
3.45	0.75	5.1	0.566	0.406	5.51	10.33	0.201	0.38	1.08
3.55	0.68	5.1	0.571	0.361	7.52	6.73	0.176	0.44	1.17
4.05	0.55	5.1	0.302	0.216	6.00	5.33	0.090	0.51	0.95
4.15	0.70	5.1	0.365	0.302	8.21	5.15	0.094	0.75	1.22
4.25	0.85	5.1	0.371	0.290	8.86	4.59	0.058	0.73	1.19
4.36	1.35	5.2	0.399	0.348	10.65	4.57	0.048	0.85	1.36
5.00	3.8	5.3							

TABLE XI.

Apr. 28, 1924. Strenuous exercise 1 min., 2.51-2.52 p.m. Subject L. 6 round trips. Water ingested every 15 min., 100 cc., 1.15-3.50 p.m. Figures represent excretion per minute.

Time.	Vol- ume.	pH	Acid	NH ₃	Total N.	$\frac{\text{NH}_3\text{-N}}{\text{Total N}}$	PO ₄	Cl	Creati- nine.
<i>p. m.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>	<i>per cent</i>	<i>cc. 0.1 M</i>	<i>cc. 0.1 N</i>	<i>mg.</i>
2.20	1.90	5.6	0.308	0.198	9.03	3.08	0.092	0.94	1.11
2.30	3.10	5.6	0.292	0.189	10.14	2.61	0.097	0.80	1.12
2.40	4.55	5.6	0.234	0.205	10.03	2.87	0.094	0.92	1.12
2.50	5.60	5.8	0.268	0.189	8.76	3.01	0.094	1.00	1.11
3.00	1.90	5.6	0.319	0.293	6.16	6.65	0.101	0.29	0.96
3.10	1.35	5.2	0.505	0.384	6.79	7.92	0.203	0.08	1.01
3.20	0.65	5.2	0.345	0.253	6.01	5.91	0.140	0.25	0.97
3.30	0.60	5.2	0.250	0.189	7.03	3.75	0.078	0.32	0.93
3.40	0.85	5.3	0.239	0.190	8.78	3.03	0.065	0.46	1.09
3.50	2.10	5.6	0.213	0.210	10.36	2.84	0.058	0.51	1.14
4.00	4.70	5.8	0.223	0.144	10.47	1.92	0.060	0.54	1.19
4.10	6.40	5.6	0.238	0.181	6.57	3.85	0.077	0.34	1.13
4.20	6.20	5.8	0.231	0.181	7.43	3.41	0.062	0.39	1.10
4.30		5.8							

TABLE XII.

May 5, 1924. Strenuous exercise 1 min., 2.50-2.51 p.m. 6 round trips. Subject L. Water ingested every 30 min., 200 cc., 1.05-3.05 p.m. Figures represent excretion per minute.

Time.	Volume.	pH	Acid.	NH ₃	Total N	$\frac{\text{NH}_3\text{-N}}{\text{Total N}}$	PO ₄	Cl
<i>p. m.</i>	<i>cc.</i>		<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>mg.</i>	<i>per cent</i>	<i>cc. 0.1 M</i>	<i>cc. 0.1 N</i>
2.10	6.80	5.8	0.317	0.318	10.69	4.08	0.191	1.75
2.20	7.70	5.8	0.359	0.275	10.44	3.69	0.201	1.70
2.30	7.20	5.8	0.291	0.208	9.09	3.20	0.143	1.25
2.40	7.00	5.8	0.305	0.225	10.56	2.98	0.145	1.23
2.50	6.28	5.8	0.274	0.299	8.86	4.70	0.149	1.14
3.00	2.28	5.5	0.285	0.348	6.36	7.70	0.163	0.36
3.10	1.25	5.1	0.467	0.474	6.00	11.09	0.247	0.20
3.20	0.85	5.0	0.415	0.270	7.31	5.10	0.228	0.44
3.30	1.35	5.1	0.285	0.232	9.07	3.57	0.111	0.51
3.40	2.80	5.3	0.233	0.237	10.48	3.17	0.094	0.74
3.50	4.08	5.8	0.233	0.218	9.14	3.35	0.090	0.63
4.00	4.25	5.8	0.207	0.190	8.42	3.16	0.085	0.63
4.10	2.60	5.8	0.207	0.187	7.59	3.47		

period of 2 hours preceding and extending through the period of exercise. In spite of these large quantities of fluid the urine volume fell to less than one-seventh of that of the fore period. The exercise was carried out for 1 minute only on a day when the temperature was 19.8°C . There was no evidence of perspiration. This together with other experiments performed in cold wet

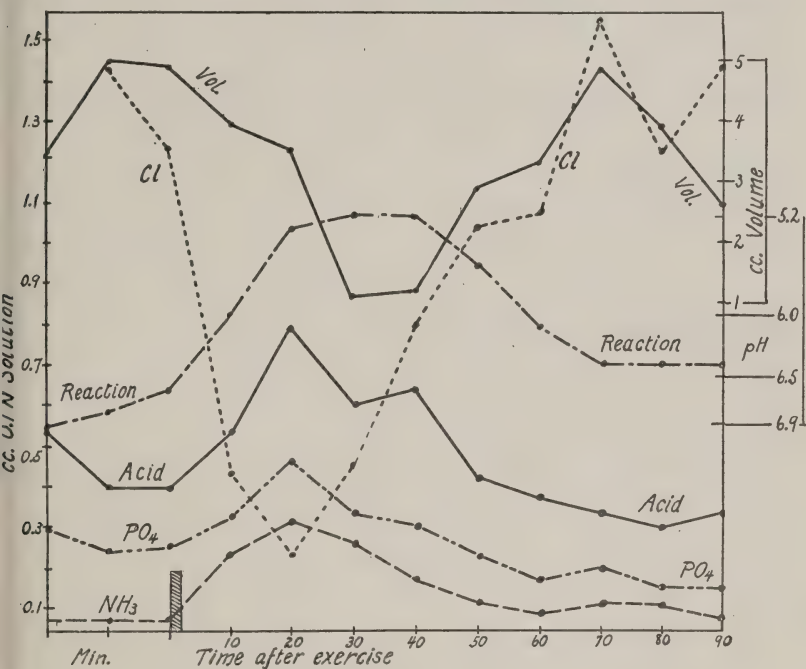


FIG. 1. Experiment 9 (see Table IX).

Since the hydrogen ion concentration rises and falls with the other acidity factors the pH of the urine is plotted inversely in Figs. 1 and 2 to show this relationship more clearly.

weather leads us to believe that the variations observed have little to do with excessive losses of fluid by way of the lungs and skin. The fact that Simpson has found increased urine volumes accompanying a slight increase in body temperature cannot be used to explain these observations.

After exercise the urine quickly became more acid and there was a rise in excretion of titratable acids and ammonia. No

matter what the reaction of the urine during the fore period the acidity increased to pH 5.5 to 5.0. In one experiment not reported a pH of 4.8 was observed. The titratable acidity was nearly doubled and the ammonia increased 2 to 5 times. The total nitrogen fell so that the rise in the percentage of ammonia nitrogen to total nitrogen was even greater. The excretion of inorganic phosphates rose while chlorides diminished. There was no significant change in the excretion of creatinine. All these changes appeared in the urines collected during the first 10 minutes after exercise and reached maxima during the second or third 10 minute

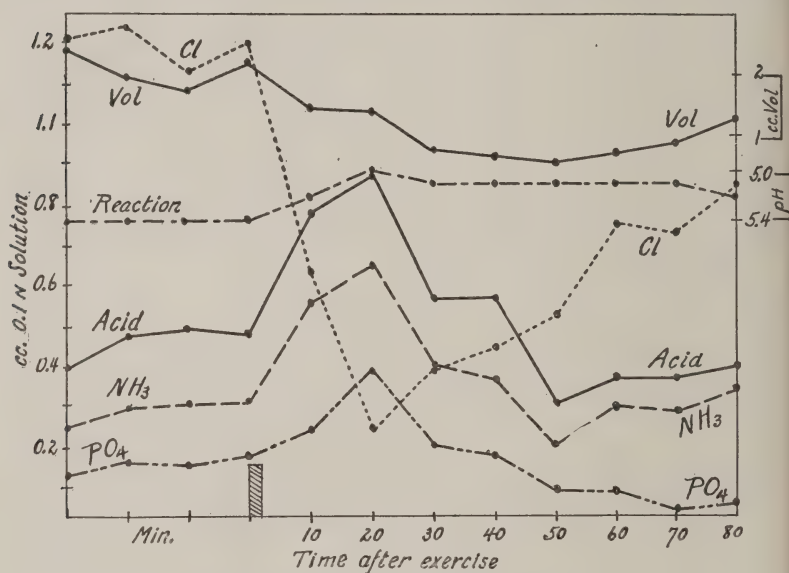


FIG. 2. Experiment 10 (see Table X).

period. Some of these variations are shown in Figs. 1 and 2 which illustrate data from Experiments 9 and 10.

The return toward normal proceeded smoothly after the extreme variation was reached. A careful comparison of the data with the variations observed in the control experiments permits a close approximation concerning the time required for the effects of the exercise to disappear.

After 1 or 2 minutes of strenuous exercise all of the variations persisted for a relatively long time and disappeared only after 30

to 90 minutes. The titratable acidity and ammonia usually returned to normal in 30 to 50 minutes, while the pH changed more slowly (50 to 90 minutes). The inorganic phosphate and total nitrogen values were back to normal in the minimum time and then swung beyond, the inorganic phosphate diminishing and the total nitrogen rising beyond the normal levels. Chlorides and urine volumes changed somewhat less rapidly.

DISCUSSION.

The variations observed are in some respects different from those found in studying long periods of strenuous exercise and suggest different explanations. In most of the older experiments the subject exercised as vigorously as possible for many hours. The most extreme variations were usually observed in subjects unaccustomed to the strenuous exertion who, therefore, became extremely fatigued and sometimes recovered only after 2 or 3 days of rest. Forms of exercise such as mountain climbing, wood chopping, ditch digging, and bicycle riding for 50 to 80 miles over hills have been resorted to.

Such experiments are recorded in papers by Engelmann (1871), Dunlop, Paton, and coworkers (1897-98), Garratt (1898-99), and Campbell and Webster (1922) where also may be found many other references to similar experiments. While the reports are not uniform the general conclusion can be drawn that during hours of severe exercise there is a loss of water and sodium chloride in the perspiration which may result in a diminished elimination of these materials for several days. As a result of the exercise the excretion of urea, phosphates, sulfates, titratable acidity, and ammonia rises. These experiments have led to the conclusion that extreme fatigue following long periods of strenuous exercise is associated with increased protein catabolism.

A few investigators have studied urines collected over sufficiently short periods so that their data and conclusions may be considered in connection with our studies. Their work may be conveniently discussed by topics.

Volume.—The short experiments of MacKeith and his collaborators (1923-24) permit conclusions to be drawn concerning the effect of exercise on the volume of urine. Diuresis was brought about by drinking tea just before the experiments. This diuresis

was quickly obliterated during rapid running, though the urine volume rose after the exercise was stopped. The loss of water by way of the skin and lungs could not account for the drop in urine volume and the authors suggest that dilatation of the cutaneous vessels and constriction of the splanchnic vessels may, together with other factors, cause the phenomenon. They found that the tense attitude assumed by a runner about to start a race caused a similar drop in urine volume.

Reaction.—Talbert (1919–20) and Endres (1922) report increased C_H of the urine after periods of $1\frac{1}{2}$ and 15 minutes of exercise. Our experiments show more extreme variations than they report.

Titratable Acidity.—Titratable acidity was studied in detail by MacKeith and collaborators who found an increased excretion after exercise. Ryffel (1909–10) reports two experiments showing the same. MacKeith and Ryffel record a few data on the excretion of ammonia which show an increased excretion after exercise.

Phosphates.—Embden and Grafe (1921) observed an increased excretion of inorganic phosphates by studying 2 hour urine collections after $2\frac{1}{2}$ to 4 hours of strenuous exercise. Blatherwick, Bell, and Hill (1924) have recently observed that less phosphate was excreted by patients when quiet in bed than when normally active.

Chlorides.—Embden and Grafe state in their study on phosphate excretion that the chlorides diminished during the 24 hour period of work but give no figures. They mention that part of the diminution is due to the loss of chlorides in the sweat, but they state that, as higher values were obtained on the following day, they think there is some type of antagonism between phosphate and chloride. The experiments of Röckemann (1922–23) indicate that this may be true. The latter investigator found that the feeding of acid sodium phosphate caused a retention of chlorides. MacKeith and coworkers report an increased excretion of chlorides after exercise.

Total Nitrogen.—Embden and Grafe state that no characteristic change was observed in the nitrogen excretion. MacKeith and coworkers report low values for total nitrogen during exercise with increases as the urine volumes rose after exercise.

Certain correlations may be made between the changes in the

composition of the urine and changes occurring in the body. The diminished urine volume after exercise may be due in the main, as MacKeith and coworkers suggest, to diminished blood flow through the kidneys. Alterations in the water distribution between plasma, corpuscles, and tissue cells due to changes in the osmotic relations may also play a rôle. Scott, Hermann, and Snell (1917) have demonstrated that muscle contractions cause water to pass from the blood into the muscle, resulting in an increased water content of muscle and a more concentrated blood. Loss of water by way of the lungs and skin can hardly account for the variations observed in our short experiments.

The changes in the reaction of the blood after exercise observed by Barr, Himwich, and Green may easily account for the diminished pH and increased ammonia and acid excretion in the urine. The acid and ammonia excretions return to normal in about the same interval of time required for the abnormal reaction of the blood to disappear. The diminution of pH of the urine and the increased excretion of ammonia indicate that the elimination of acid metabolic products was being accomplished with the minimum loss of base from the body. The effectiveness of these mechanisms, however, was not great. In Experiment 9 (Table IX) an increased excretion of about 0.8 mm of ammonia resulted from the exercise and about 0.9 mm of base was made available by eliminating the phosphate in urine with a lower pH. Judging from similar experiments reported in the next paper on the same individual from 5 to 16 mm of lactic acid were excreted combined with base. The body, therefore, lost base.

The same mechanisms undoubtedly remain in force to bring about a retention of base after the large quantities of acid had been excreted. Such an adjustment probably accounts for the unusually low pH values and high ammonia excretion observed in the control experiment of Subject W. (Table VI) carried out 3 hours after an experiment involving two periods of strenuous exercise in which 2.16 gm. of lactic acid were excreted with 24 mm of base.

Embden and Adler's (1922) experiments on isolated muscles would indicate that the increased excretion of phosphate is due to the liberation of phosphoric acid in the active muscle and its diffusion into the blood stream and thence into the urine. The greatly diminished excretion during the later periods in the experi-

ments suggests that the recovery after muscular exercise involves the retention and utilization of phosphates, possibly for synthesizing the "lactacidogen" of muscle. Embden and Lange's (1923) experiments also point to a diffusion of chloride into the active muscle. This observation, together with other shifts which probably occur due to change in reaction of the body fluids, may account for the diminished chloride excretion after exercise. It is improbable that appreciable quantities of chlorides were lost by way of the skin. The retention of water by the body necessitating a retention of chlorides as well as urea should not be overlooked.

The relationship between the changes in the composition of the urine and the composition of the blood which may be inferred from the correlations mentioned above demonstrates how rapidly and efficiently the kidney responds to assist in the regulation of the neutrality and osmotic pressure in the body. A study of the variations of water, phosphate, and chloride concentrations in blood and tissues may throw further light on these relationships.

SUMMARY.

The effect of short periods of strenuous exercise on the composition of the urine was studied. For this purpose, urine was collected in 10 minute periods. There were observed, following exercise, a decrease in urine volume, and an increase in hydrogen ion concentration, and acid and ammonia excretion. The elimination of phosphates rose while that of chlorides fell. Each of these changes reached its maximum within 20 to 30 minutes and was followed by normal values within 40 to 90 minutes after 1 or 2 minutes of exercise.

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THE EXCRETION OF LACTIC ACID IN THE URINE AFTER MUSCULAR EXERCISE.*

BY S. H. LILJESTRAND AND D. WRIGHT WILSON.

(From the Department of Physiological Chemistry, School of Medicine,
University of Pennsylvania, Philadelphia.)

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The classic work of Fletcher and Hopkins (1906-07) demonstrated conclusively that lactic acid is formed in muscle during contraction. Since that time lactic acid production has been proved to be of fundamental importance in muscle physiology (Hill and Meyerhof, 1923; Embden, Griesbach, and Schmitz, 1914-15). Hill's studies on oxygen utilization led him to conclude that large quantities of lactic acid are formed in muscle during strenuous exercise. Free diffusion into the blood may result in concentrations of over 100 mg. of lactic acid per 100 cc. of blood, about five times the normal value (Hill and Lupton, 1922-23; Barr, Himwich, and Green, 1923). With such abnormal quantities in the blood it is natural to assume that lactic acid will pass through the kidney and be excreted in the urine. Ryffel (1909-10) and Feldman and Hill (1911) found from 60 to 450 mg. excreted in 30 minutes after short periods of running. If Hill and Lupton (1922-23) are correct when they calculate that from 20 to 90 gm. of lactic acid may be formed in the body during a few minutes of strenuous exercise, the amounts found in the urine seem remarkably small. Various facts, however, suggest explanations for such a relationship. Relatively inactive muscles are able to absorb lactic acid from the blood in which the concentration has been raised by exercise (Barr and Himwich, 1923). Other tissues of the body may perhaps function in the same way. After short periods of strenuous exercise, lactic acid disappears

* A preliminary announcement was made in the Proceedings of the Society for Experimental Biology and Medicine (Liljestrand, S. H., and Wilson, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 426).

rapidly owing to oxidation and synthesis so the time in which excretion can take place is short. The ability of the body to utilize large quantities of lactic acid is shown by the experiments of Nencki and Sieber (1882) who fed 20 gm. of sodium lactate a day for 4 days to a diabetic without being able to detect a trace of lactic acid in the urine and by the experiments of Haggard and Henderson (1920) who injected large quantities of lactic acid without seriously diminishing the alkaline reserve of the blood.

It should be emphasized that Hill's work shows that exercise sufficiently mild to permit continuous performance for a considerable time (*i.e.* $\frac{1}{2}$ to 1 hour) does not lead to a continuous piling up of lactic acid. The severity of the sustained effort seems to be such that a "steady state" is reached where lactic acid is disposed of as fast as it is formed. This may account for the fact that only small quantities of lactic acid have been found in the urine after long periods of exercise. Jerusalem (1908) found 33 mg. in 250 cc. of urine after mountain climbing and Campbell and Webster (1922) found 61 mg. in urine after 5 hours of hard work.

EXPERIMENTAL.

In order to obtain additional data concerning the excretion of lactic acid in urine after strenuous exercise several experiments were carried out on young men using 2 to 3 minute periods of exercise (stair running). The urine was collected in 10 or 15 minute periods to permit the rate of excretion to be followed in some detail. The experiments were usually carried out in the morning, after breakfast, at which both subjects drank coffee. After a suitable fore period, the subject ran up and down one flight of stairs as rapidly as possible, moving his arms vigorously with each step. The collection of urine was continued in most experiments for 50 to 60 minutes.

After the urines were measured they were analyzed for lactic acid by the method of Clausen (1922). It is recognized that other materials may be determined as well as lactic acid, but as the urines were free from protein, acetone, and acetoacetic acid, there seems to be little reason for doubting that the increased values observed after exercise were due to the excretion of lactic acid itself. The isolation of 400 mg. of zinc lactate from a portion

of the urine from one experiment demonstrates the presence of a considerable quantity of lactic acid. From 0.5 to 2 cc. of urine were extracted with ether and the analysis carried out as out-

TABLE I.

Lactic acid in urine. Subject W. Mar. 31, 1924. Stair running, 2 min.

Time.	Urine volume.	Lactic acid.	Lactic acid concentration.	Remarks.
<i>a. m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg. per 100 cc.</i>	
9.00- 9.15	16.3	2.6	16	
9.15- 9.30	23.0	200	870	Exercise 9.15-9.17 a.m.
9.30-10.00	26.5	276	1040	

Total lactic acid excreted, due to exercise, 470 mg.

TABLE II.

Lactic and phosphoric acids in urine. 10 min. periods. Subject W. Apr. 14, 1924. Stair running, 2 min. (8 round trips).

Time.	Urine volume.	Lactic acid.	Lactic acid concentration.	PO ₄	Remarks.
<i>a. m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg. per 100 cc.</i>	<i>cc. 0.1M</i>	
9.17- 9.27	17.8	1.9*	11	1.15	Drank 200 cc. water. Room temperature 20°C.
9.27- 9.37	28.0	2.4*	9	1.36	
9.37- 9.47	29.0	1.9*	7	1.12	
9.47- 9.57	18.7	126	670	1.68	Exercise 9.47-9.49 a.m.
9.57-10.07	34.0	426	1250	2.94	
10.07-10.17	15.5	145	930	2.14	
10.17-10.27	9.1	29	320	1.06	
10.27-10.37	11.8			0.88	
10.37-10.47	9.5	1.7*	18	0.57	
10.47-10.57	11.5	1.6*	14	0.43	

Total lactic acid excreted due to exercise 720 mg.

All specimens gave negative tests for protein, acetoacetic acid, and acetone.

* Single analysis.

lined by Clausen. Distillation with sulfuric acid instead of permanganate was used. We found that considerable experience was necessary before suitable analyses could be obtained on pure zinc lactate solutions. The part of the procedure which was most

difficult to control was the oxidation and distillation into the sodium bisulfite solution, using a vigorous current of air. Most

TABLE III.

Lactic acid in urine. 15 min. periods. Subject Li. Apr. 3, 1924. Stair running, 3 min. (10 round trips).

Time.	Urine volume.	Lactic acid.	Lactic acid concentration.	Remarks.
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg. per 100 cc.</i>	
9.30-9.45	13.0	1.4	11	9.50 a.m. drank 200 cc. water.
9.45-10.00	15.0	1.7	11	
10.00-10.15	18.5	129	700	Exercise 10.00-10.03 a.m.
10.15-10.30	17.5	56	320	
10.30-10.45	34.5	3.5	11	
10.45-11.00	31.5	1.8	6	
11.00-11.15	32.5	2.3	7	

Total lactic acid excreted due to exercise 180 mg.

All specimens gave negative tests for protein, acetoacetic acid, and acetone.

TABLE IV.

Lactic acid in urine. 10 min. periods. Subject Li. May 26, 1924. Stair running, 2 min.

Time.	Urine volume.	Lactic acid.	Lactic acid concentration.	Remarks.
<i>p.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg. per 100 cc.</i>	
2.00-2.10	9.0	1.3	14	Exercise 2.20-2.22 p.m.
2.10-2.20	10.0	1.6	16	
2.20-2.30	7.0	47	670	
2.30-2.40	8.3	86	1040	
2.40-2.50	5.0	12	240	
2.50-3.00	3.6	1.2	33	
3.00-3.10	6.8	1.8	26	
3.10-3.20	6.0	1.8	30	

Total lactic acid excreted due to exercise 140 mg. Negative tests for protein except trace in first specimen after exercise; this and the following specimen gave negative tests for acetoacetic acid.

of the trouble was obviated by introducing a thermometer into the distilling tube so that the bulb was beneath the surface of the

liquid. If the temperature of the boiling liquid was maintained between 145° and 150° during the distillation a quantitative recovery of the aldehyde could be made. The temperature of the boiling liquid was from 5° to 10° lower than that of the metal bath outside. Water was regularly added by way of the capillary intake tube to keep the concentration of H_2SO_4 approximately 1:1 and thereby eliminate charring. The distillation was continued for 1 hour. The blank for 0.01 N iodine was 0.07 cc. and for 0.001 N iodine, 0.7 cc. The following qualitative tests were made on many of the specimens of urine: protein, heat, and acetic acid; acetoacetic acid, ferric chloride; acetone, Rothera's test.

DISCUSSION.

The normal urines when analyzed by Clausen's method gave results ranging from 1 to 2.5 mg. of lactic acid excreted in 10 minutes. These figures agree with those of Ishihara (1913), Dapper (1913), and Clausen (1922). As lactic acid has never been isolated from normal urine it is doubtful whether these analyses represent lactic acid. After exercise the excretion rose suddenly, reaching a maximum in the second 10 minute period. During this period the excretion ranged in different experiments from 85 to 630 mg. After 20 minutes the excretion fell rapidly and at the end of 30 to 50 minutes lactic acid had probably disappeared from the urine as normal figures were again obtained. Allowing for normal figures we have calculated that a total of 140 to 1370 mg. of lactic acid were excreted after these short periods of strenuous exercise. In all of the experiments Subject W. excreted more lactic acid than Subject Li. after the same amount of work.

A few experiments were carried out in which a second period of exercise was undertaken 30 or 40 minutes after the first to determine whether the lactic acid excretion would be influenced by the previous exercise. The variations in the urine and the total quantity of lactic acid excreted were similar to those found after a single period of exercise. The maximum quantity of lactic acid excreted in the two periods of one experiment was 2.16 gm.

The concentration of lactic acid in the normal urines ranged from 6 to 21 mg. per 100 cc. After exercise the maximum con-

centration varied from 700 to 1840 mg. per 100 cc. In some of these urines the concentration of lactate may have been higher than any other solid constituent of the urine.

The concentration of lactic acid in the urine as well as the quantities excreted reached maxima, in most instances, during the second 10 minute period and then decreased rapidly, reaching normal values in from 30 to 50 minutes after exercise. The rise and the return to normal proceed at about the same rate as that

TABLE V.

Lactic and phosphoric acids in urine. 10 min. periods. Subject Li. Apr. 30, 1924. Stair running, two periods of 2 min. each (8 round trips each time).

Time.	Urine volume.	Lactic acid.	Lactic acid concentration.	PO ₄	Remarks.
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg. per 100 cc.</i>	<i>cc. 0.1 M.</i>	
8.30- 8.40	10.0	1.3	13	1.56	Drank 200 cc. water.
8.40- 8.50	6.5	1.0	15	0.81	
8.50- 9.00	12.5	103	830	1.44	Exercise 8.50-8.52 a.m.
9.00- 9.10	15.2	131	860	2.04	Slight perspiration.
9.10- 9.20	7.7	26	340	1.03	
9.20- 9.30	12.1	76	630	1.16	Exercise 9.20-9.22 a.m.
9.30- 9.40	8.1	72	890	1.34	Moderate perspiration.
9.40- 9.50	8.5	15	176	0.46	Drank 100 cc. water.
9.50-10.00	8.5	2.1	25	0.46	
10.00-10.10	11.3	1.4	12	0.39	
10.10-10.20	13.3	1.3	10	0.36	
10.20-10.30	17.0	1.2	7	0.27	

Total lactic acid due to exercise: first period, 250 mg.; second period, 160 mg. Single analyses only.

observed for other urinary constituents reported in the previous paper (Wilson, Long, Thompson, and Thurlow, 1925). In two experiments, the phosphate excretion was again followed. Hill, Long, and Lupton (1924) have found that the concentration of lactic acid in the blood rises rapidly with exercise and reaches a maximum probably in 5 minutes or less after its cessation. Their data indicate, however, that the return to normal is gradual and not complete for 1 to 1½ hours. Whether the difference in time

necessary to reach normal levels in blood and urine is due to individual variations or to imperfections inherent in the quantitative method used (Long, 1923-24) or to a definite threshold for lactic acid excretion is impossible to state at present.

The elimination of 1 to 2 gm. of lactic acid involves the removal from the body of 11 to 22 mm of base because lactic acid is practically completely combined with base at the reaction of the urine. This is an appreciable quantity of base to be furnished in addition

TABLE VI.

Lactic acid in urine. 10 min. periods. Subject Li. June 26, 1924. Stair running, two periods of 2 min. each (8 round trips each time).

Time.	Urine volume.	Lactic acid.	Lactic acid concentration.	Remarks.
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg. per 100 cc.</i>	
8.00- 8.10	6.5	1.2	18	Drank 300 cc. water. Room temperature 21.7°C.
8.10- 8.20	7.5	1.6	21	
8.20- 8.30	9.0	94	1040	Exercise 8.20-8.22 a.m. Considerable perspiration.
8.30- 8.40	10.7	128	1200	
8.40- 8.50	6.0	53	880	
8.50- 9.00	10.7	155	1450	Exercise 8.50-8.52 a.m. Considerable perspiration.
9.00- 9.10	10.7	197	1840	
9.10- 9.20	7.2	92	1280	
9.20- 9.30	5.2	10	192	
9.30- 9.40	6.4	2.5	39	
9.40- 9.50	5.9	1.4	24	
9.50-10.00	8.8	2.1	24	

Total lactic acid due to exercise: first period, 270 mg.; second period, 450 mg. Single analyses only.

to the quantity necessary to form the salts of hydrochloric, sulfuric, and phosphoric acids. Replacement of lost base is most simply accomplished by an increased CH of the urine which permits the excretion of phosphate with the minimum amount of base, and by the increased elimination of ammonia. Such an adjustment was probably at work in the control experiment of Subject W. on May 30 (reported in the previous paper), carried out in the afternoon after a double exercise experiment in the morning. The pH of the urine was 5.3 whereas in all other

experiments in which the same individual was the subject the pH was found to range between 6.2 and 7.4 at the same time of day. In the morning 2.16 gm. of lactic acid had been excreted with the removal of 24 mm of base.

In looking over the literature, we failed to find that the lactic acid obtained from urine after exercise had been identified as dextro- or sarcolactic acid. It is known to be formed in muscle during exercise and to be present in urine in various pathological

TABLE VII.

Lactic acid in urine. 10 min. periods. Subject W. May 30, 1924. Stair running, two periods of 2 min. each (8 round trips each time).

Time.	Urine volume.	Lactic acid.	Lactic acid concentration.	Remarks.
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg. per 100 cc.</i>	
9.10- 9.20	20.2	1.6	8	Drank 100 cc. water.
9.20- 9.30	13.8	1.4	10	
9.30- 9.40	23.0	197	860	Exercise 9.30-9.32 a.m. Slight nausea and dizziness with perspiration.
9.40- 9.50	48.5	632	1300	
9.50-10.00	25.8	410	1590	
10.00-10.10	13.5	139	1030	Exercise 10.10-10.20 a.m.
10.10-10.20	14.3	147	1030	
10.20-10.30	30.0	396	1320	
10.30-10.40	17.0	166	980	
10.40-10.50	9.8	71	720	
10.50-11.00	10.0	18*	180	
11.00-11.10	10.0	2.8*	28	

Total lactic acid due to exercise: first period, 1370 mg.; second period, 790 mg.

* Single analysis.

conditions, but the few investigators who have isolated lactic acid (usually as the zinc salt) from urine after exercise or after strychnine convulsions have failed to prove that they had the dextro form. The active form may be identified by the analysis of the hydrated crystals (zinc dextro-lactate crystallizes with 2 molecules of water of crystallization and inactive zinc lactate with 3) or by determining the specific rotation. As the water content of the crystals is said to be variable (Fletcher and Hopkins,

1906-07), many investigators have been satisfied to analyze the dried crystals for zinc to identify their preparations. Spiro (1877-78) isolated zinc lactate from tetanized animals and obtained a small quantity of impure material from the urine of men after mild exercise. Colasanti and Moscatelli (1887) isolated zinc lactate from the urine of soldiers after a forced march. In neither investigation were the data sufficient to demonstrate that the zinc salt of dextro-lactic acid was obtained.

After determining the lactic acid in urine following exercise, the remaining urine from one experiment was extracted, as in the quantitative procedure, and zinc lactate prepared by heating the ether extract with water and zinc carbonate. After evaporating off the ether and filtering, the solution was concentrated to a small volume and allowed to stand. The zinc lactate obtained was recrystallized once from water and analyzed.

Water found, 13.2 per cent. Theoretical for zinc sarcolactate, 12.9 per cent. Theoretical for inactive zinc lactate, 18.2 per cent.

0.3602 gm. of dried salt (representing 0.4153 gm. of hydrated salt) was dissolved in water and made up to 10 cc. Using a 1 dm. tube, $\alpha = -0.31^\circ$ at about 20° . $[\alpha]_D = -7.5^\circ$ for hydrated salt. For a 4.18 per cent solution (Hoppe-Seyler and Araki, 1895), $[\alpha]_D = -7.51^\circ$.

Zinc found, 26.1 per cent of anhydrous salt. Theoretical for zinc sarcolactate, 26.8 per cent.

A few mg. of the dried salt gave a strongly positive reaction with Hopkins' thiophene test.

The isolation of zinc sarcolactate demonstrates that it is the dextro- or sacrolactic acid which is present in urine after strenuous exercise.

SUMMARY.

The excretion of lactic acid in urine after 2 to 3 minutes of strenuous exercise was studied in 10 and 15 minute periods. The excretion reached maxima varying from 86 to 630 mg. during the second 10 minute period after exercise. Thereafter, the elimination diminished and normal values were reached in 30 to 50 minutes.

The total quantity of lactic acid excreted after one period of

exercise varied from 140 to 1370 mg. A second period of exercise following shortly after the first yielded similar quantities.

The lactic acid was shown to be the dextro form.

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THE INFLUENCE OF ORALLY ADMINISTERED CALCIUM SALTS ON THE SERUM CALCIUM OF NORMAL AND THYREOPARATHYROID DOGS.

By AXEL M. HJORT.

(From the Department of Chemical Research, Parke, Davis and Company, Detroit.)

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Calcium is a very important constituent of the body tissues, not only in making up the bony skeleton but also in maintaining certain nervous and muscular equilibria. Calcium equilibrium is well maintained in health by the ordinary foods. The calcium salts are absorbed, and transported through the body in various forms. It may be combined as bicarbonate, phosphates, proteinates, and perhaps hexose phosphates as suggested by Robison (1). The preservation of equilibrium in the tissues is ample evidence of calcium absorption in the alimentary canal. The actual process of absorption has led to considerable discussion depending on the acceptance of certain governing factors. An excess of phosphate or alkali would naturally limit absorption by converting the calcium into relatively insoluble salts. Givens (2) noted an increase in calcium elimination in the stools when fats and fatty acids were poorly utilized. He (3) also demonstrated that the form in which calcium was ingested influenced absorption, for the amount of calcium absorbed from milk exceeded that from calcium lactate when both were given in like quantity as calculated by their calcium contents. In health and with ordinary foods the required amount of calcium is absorbed in spite of the presence of interfering agencies. Such is not the case in certain diseases.

In rachitis, in which Schabad (4), Schloss (5), and Orr (6) have shown a calcium and phosphorus deficiency, these two elements are poorly absorbed. Zucker and Matzner (7) in their observations on the pH of the intestinal contents of rachitic

rats before and after treatment with cod liver oil found a possible explanation for the impaired absorption. In the active untreated state they noted that the feces were quite alkaline, whereas after treatment and coincident with improvement the intestinal contents became acid. The former condition impairs, and the latter facilitates absorption of lime salts. The work of Babbott (8) in a way confirms the foregoing observations. He and his coworkers found in human rickets an impaired acid-excreting function of the stomach which improved with convalescence. They believe that the gastric acidity influences absorption of calcium by its effect on the reaction of the upper intestine. According to Steenbock (9), calcium salts which are soluble in acid media are absorbed sufficiently for the body needs. Orr (6) observed an increase in the calcium contents of the urine in human rickets during the transition from the active to the cured stages. All of the above phenomena may be explained by the observations of Zucker and Babbott.

Since the advent of microchemical blood analyses, calcium has been given its share of consideration. Several fairly accurate methods of analysis have been devised, and through their medium calcium has been estimated in the blood in health and disease. Thus certain normal standards have been established which serve as a basis for experimentation. The effect of orally administered calcium salts upon the normal and abnormal blood calcium values has been investigated by several writers. Clark (10) failed to increase the blood calcium of rabbits by feeding calcium lactate. Denis and Minot (11) could not raise the plasma calcium of several human subjects by feeding 6 gm. of calcium lactate daily for a period of 10 days. Kramer and Howland (12) failed to note a rise in the serum calcium of normal rats by calcium feeding, but observed an increase in rachitic rats. Jansen (13) studied the blood calcium changes in a normal human subject after feeding various calcium salts, and noted an increase which varied with the salt employed. Salvesen (14) observed a 20 per cent increase in the serum calcium of one of two dogs receiving 7.5 gm. of calcium chloride orally, but found no change in the other. From the foregoing, it is obvious that calcium salts administered orally may in some cases, at least, increase the blood calcium values. Because of the interest in

this subject lately, the present writer endeavored to study the effects of several calcium salts upon the serum calcium of normal and thyreoparathyroprivic dogs. The results of this study are recorded below.

EXPERIMENTAL.

I. Preliminary Comment.

Several microchemical calcium procedures were tried, and finally the Kramer-Tisdall (15) blood serum method was adopted, and employed throughout the experimental work herein recorded. It was chosen because of its relative simplicity, uniformity in results, and an accuracy which is all that can be expected of such a microchemical estimation. It is essentially an adaptation of the Halverson-Bergeim (16), de Waard (17), and Clark (18) procedures. In general, it consists in precipitation of the calcium by means of ammonium oxalate, removal of the excess oxalate by repeated washing, and estimation of the calcium by titration with $N/100$ potassium permanganate in acid solution.

The entire procedure was performed in centrifuge tubes, and the supernatant liquids were syphoned off after spinning. The potassium permanganate titrations were made with a 1 cc. glass stop-cocked burette graduated in 0.01 cc. Duplicate analyses were made in all cases, and as a rule checked within 0.01 cc. Occasionally 0.02 cc. differences were encountered, but rarely greater. When the deviations exceeded 0.02 cc., the results were disregarded. A variation of 0.02 cc. of $N/100$ $KMnO_4$ represents a difference of 0.4 mg. of calcium per 100 cc. of serum.

Some criticism has been directed against this type of procedure because of the relative instability of $N/100$ potassium permanganate solutions. The writer has found, however, that if this solution is carefully prepared according to the directions of Halverson and Bergeim (19), it may be preserved as long as 8 to 10 weeks with a final maximum depreciation of but 1 per cent.

In several experiments, both when water alone, and when calcium salts in 5 per cent solution were administered to normal and thyreoparathyroprivic dogs, the total blood solids were estimated gravimetrically parallel with the serum calcium

analyses. The results showed either no change or a slight dilution of the blood. The blood was drawn at 1 or 2 hour intervals during the 6 hour period under which the experiments were conducted. The calcium salt solutions were given by a stomach tube immediately after the initial blood drawing.

All dogs with the exception of Nos. XIX and XXXIII of Section III were fed on a constant aciduric diet consisting of boiled beef heart, boiled rice, lactose, and milk as described by Dragstedt (20). Dog XIX ran away for a week, and returned on the day of experiment. Dog XXXIII was chosen as a control to study the absorption of calcium in animals on an ordinary "scraps" diet. In every case, with the above exceptions, the aciduric diet was commenced at least 2 weeks before the calcium absorption experiments. All dogs were fasted about 16 hours before commencement of experiments, and received neither food nor additional water during the course of the observations. Female dogs were employed throughout, and practically all were young adults.

II. Effect of Water Ingestion on the Serum Calcium of Normal Dogs.

As the calcium salts were to be given in aqueous solutions, it was important to know beforehand what influence water alone would have on the serum calcium when administered in quantities equivalent to that used as a vehicle for the salts. In pursuing this information Dogs XIX, XXII, and XXI were employed. The first two were of practically the same weight while the last was twice their size. Lukewarm tap water was administered orally by a stomach tube immediately after the first blood collection. Blood solids were determined simultaneously with the drawing of blood for calcium analyses in two of the three dogs. There was found to be a slight dilution of the blood in these cases. Details of the calcium studies are recorded in Chart 1.

III. Effect of Calcium Lactate Ingestion on the Serum Calcium of Normal Dogs.

Calcium lactate, 1.5 gm. per kilo of body weight, was administered by stomach tube in 5 per cent solution, with lukewarm

tap water, to each of seven dogs. The blood was collected in each case immediately before giving the calcium solutions, and at 1 or 2 hour intervals thereafter up to 6 hours, at which time the observations were discontinued. The calcium lactate was retained by all, neither emesis nor diarrhea following. The details of these experiments are given in Chart 2.

IV. Effect of Ingestion of Some Other Calcium Salts on the Serum Calcium of Normal Dogs.

The influence of some other calcium salts on the serum calcium values of normal dogs was studied for comparison with the

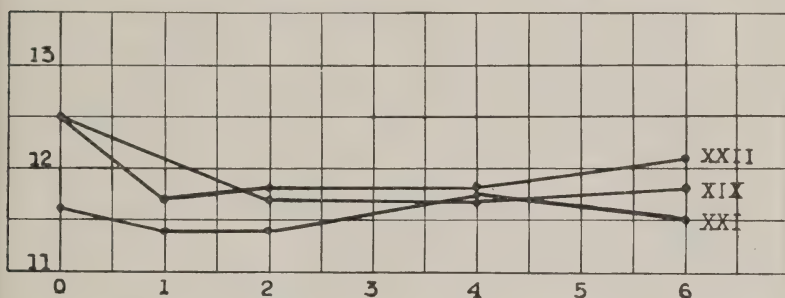


CHART 1. Effect of water administered *per os* on serum calcium of normal dogs.

Abscissa, time in hrs. after water administration.

Ordinate, serum calcium in mg. per 100 cc.

Dog XIX,	weight	9.0 kilos,	250 cc. water	<i>per os</i> .
" XXI,	"	20.0 "	600 "	" " "
" XXII,	"	10.0 "	150 "	" " "

results obtained with calcium lactate. According to Jansen, calcium lactate induced less increase in the serum calcium than did salts such as the chloride, glycerophosphate, and bicarbonate. This does not seem plausible in the case of the last two for these are less soluble than the lactate, hence the observations reported below include the above salts and in addition calcium carbonate. The amount of each administered was so calculated as to contain calcium equivalent to 1.5 gm. of calcium lactate or 0.2727 gm. of the oxide per kilo of body weight. There is one exception in the instance of the bicarbonate which could not be dissolved in that amount.

Calcium chloride was given by stomach tube to each of five dogs, but only one could retain it, emesis occurring within $\frac{1}{2}$ hour after its administration. Apparently the chloride in 5 per cent solution is more irritating to the gastric mucosa than is the lactate.

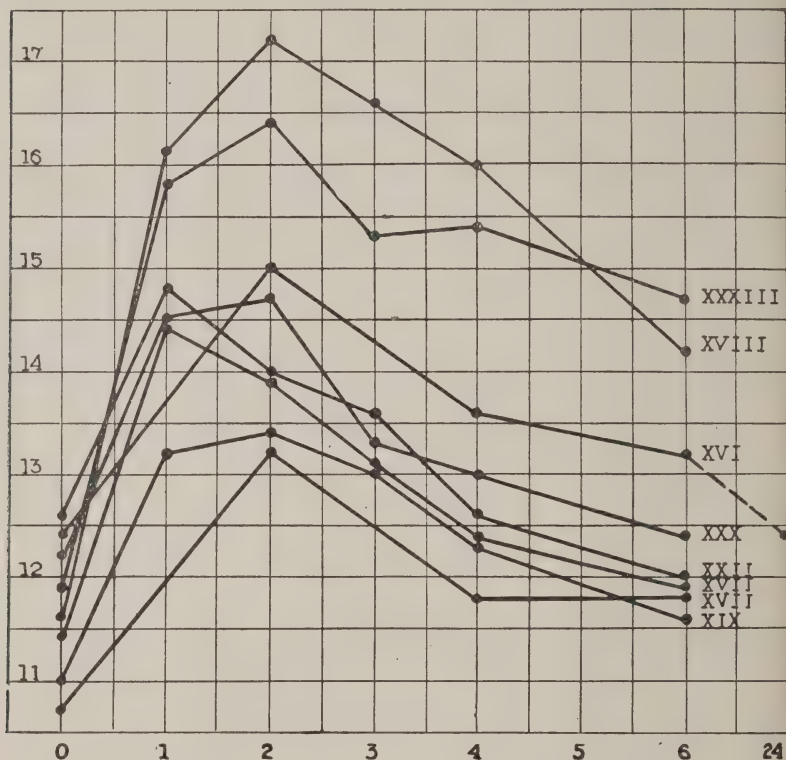


CHART 2. Effect of calcium lactate administered orally on serum calcium of normal dogs.

Abscissa, time in hrs. after Ca administration.

Ordinate, serum calcium in mg. per 100 cc.

Dog XVI,	weight 15.7 kilos.
" XVII,	" 11.0 "
" XVIII,	" 10.0 "
" XIX,	" 9.0 "
" XXII,	" 10.0 "
" XXX,	" 14.0 "
" XXXIII,	" 11.4 "

Calcium lactate 1.5 gm. per kilo in 5 per cent solution with tap water was administered orally to all the above dogs.

Calcium glycerophosphate, calcium carbonate, and calcium bicarbonate were respectively administered to each of two dogs and in no case was gastric irritation evident.

Calcium bicarbonate because of the difficulty of its preparation and instability was made by two processes, and administered immediately after completion. In the first case, it was prepared by supersaturating a solution of calcium hydroxide with carbon dioxide under increased pressure. In the second instance, calcium chloride and sodium bicarbonate solutions were mixed.

The details of the above observations are recorded in Chart 3.

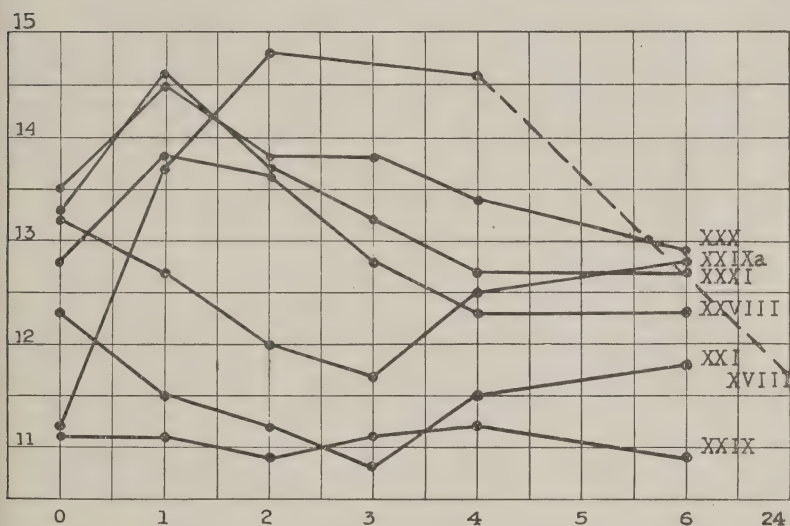


CHART 3. Effect of some other calcium salts administered orally on the serum calcium of normal dogs.

Abscissa, time in hrs. after Ca administration.

Ordinate, serum calcium in mg. per 100 cc.

Dog XVIII,	weight 10.0 kilos,	CaCl ₂ 0.54 gm. per kilo,	5 per cent solution.
" XXXI,	" 14.5 "	Ca glycerophosphate 1.023 gm. per kilo.	
" XXX,	" 14.0 "	" " 1.023 " " "	
" XXI,	" 20.0 "	Ca(HCO ₃) ₂ 2.8 gm. 660 cc. H ₂ O.	
" XXIXa,	" 9.5 "	" " 200 " "	
" XXVIII,	" 9.0 "	CaCO ₃ 0.487 gm. per kilo, 200 cc. H ₂ O.	
" XXIX,	" 9.5 "	" 0.487 " " " 300 " "	

CaCl₂ 5.23 gm., NaHCO₃ 7.79 gm. High initial values due to high summer temperature.

V. Effect of Orally Introduced Calcium Lactate on the Serum Calcium of Thyreoparathyroprivic Dogs.

The effect of calcium lactate on the serum calcium of dogs in various stages of hypoparathyroidism was also studied. Luckhardt (21) found that at least 1.5 gm. of this salt per kilo of

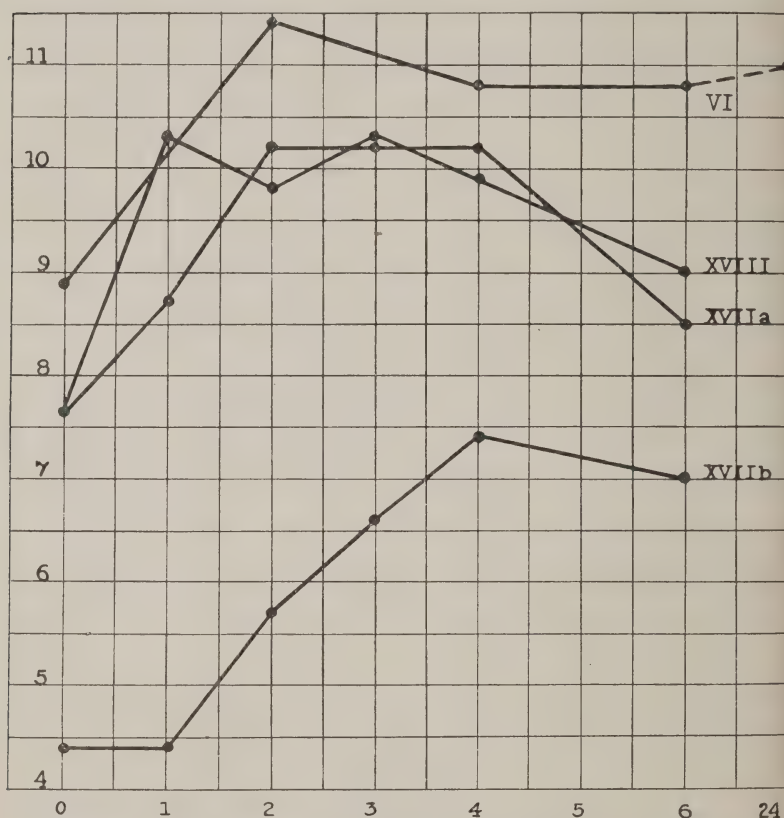


CHART 4. Effect of calcium lactate administered orally on the serum calcium of thyreoparathyroidectomized dogs.

Abscissa, time in hrs. after Ca administration.

Ordinate, serum calcium in mg. per 100 cc.

Dog VI,	weight 10.0 kilos.
" XVII, a and b,	" 11.5 "
" XVIII,	" 12.0 "

Dog XVIIa in mild tetany; Dog XVIIb in severe tetany.

body weight were required to control tetany. He did not study serum calcium changes in his cases. The present writer believes that orally administered calcium salts control tetany by their influence on the blood calcium, and to support this view the calcium absorption experiments were extended to thyreoparathyroprivic dogs.

Complete thyreoparathyroidectomy was performed on three dogs, and serum calcium analyses were made, when indicated, to follow the progress of the disturbed glandular condition. All three dogs developed tetany within 36 hours after operation, and were kept alive at critical periods by orally administered calcium lactate. In the case of Dog VI the experiment was conducted while slight hypocalcemia prevailed. In Dog XVII one experiment was performed during a mild tetanic attack and another during a very severe one. Dog XVIII had mild tetany when it served for experiment.

Details of these observations appear in Chart 4.

DISCUSSION.

A. On examination of Chart 1, it is apparent that the only influence the water content of the calcium salts can exert is that of lowering the serum calcium. This lowering is very slight and may be due to a dilution of the blood as is indicated by the decrease in blood solids.

B. Calcium lactate in doses of 1.5 gm. per kilo of body weight invariably induces an increase in the serum calcium of normal dogs when administered orally in 5 per cent solutions as is shown in Chart 2. The serum calcium increase ranges between 2.2 and 5.6 mg. per 100 cc. or 17.4 and 48.2 per cent. One dog studied on two different occasions showed approximately the same increase each time. Blood solids determined simultaneously with the serum calcium in two dogs show evidence of a slight dilution of the blood, as when water alone is given *per os*. The maximum serum calcium increase occurs as a rule in 1 to 2 hours after ingestion of the calcium lactate. At the end of 6 hours the serum calcium closely approaches its preingestion value. Calcium, therefore, is rapidly eliminated from the blood after absorption.

When calcium lactate is given in doses of 0.75 gm. per kilo of body weight it may still be absorbed rapidly enough to increase the serum calcium, but is not constant in action. In some cases the serum calcium has remained unchanged after oral administration of this smaller dose, whereas in others it has increased as high as 18 per cent. For the sake of brevity, the results with small doses are not charted.

In one dog calcium lactate, 3.0 gm. per kilo of body weight, was given orally. Emesis and severe diarrhea resulted.

C. In Chart 3 one sees for comparison with calcium lactate the effect of some other calcium salts on the serum calcium of normal dogs. Some high initial serum calcium values were encountered due no doubt to the hot spell prevailing at the time. As proof for this surmise attention is called to the curves for Dog XXIX, numbered XXIX and XXIXa respectively. During the hot spell Experiment XXIXa was performed, initial serum calcium 13.2mg.; a week later during moderate weather the same dog had an initial value of 11.1 mg. (No. XXIX). Furthermore, the potassium permanganate used for titration was the same on both occasions, and standardization showed no change in its normality.

The curve representing calcium chloride, 0.54 gm. per kilo of body weight in 5 per cent solution, shows a serum calcium increase of 3.6 mg. or 32.1 per cent. It closely simulates the calcium lactate curves. Because of the irritating nature of calcium chloride, it is less desirable for oral administration than is the lactate.

Calcium glycerophosphate, 1.023 gm. per kilo of body weight, an equivalent of 1.5 gm. of the lactate, increased the serum calcium 1.1 mg., in one dog, and 1.3 mg. in another, or 8.26 and 9.8 per cent respectively. The glycerophosphate is, therefore, less efficient than the lactate in raising the serum calcium.

Calcium carbonate, 0.487 gm. per kilo of body weight, a calcium equivalent of 1.5 gm. of the lactate, failed to affect the serum calcium of one dog, and increased that of another 1.0 mg. or 7.8 per cent. Calcium carbonate may, therefore, be absorbed rapidly enough to raise the serum calcium level slightly. This case possibly suggests the importance of a normal gastric acidity in facilitating the absorption of calcium from a relatively insoluble salt.

Jansen as quoted above found calcium bicarbonate the most serviceable of the calcium salts for inducing an increase in the serum calcium in man. He also found calcium lactate the least efficient in his series. Calcium bicarbonate as prepared by the present writer was given to each of two dogs. In neither case could the serum calcium level be raised. In fact there was a tendency toward a diminution, an explanation for which is wanting. Calcium bicarbonate in dogs, therefore, appears to have no advantage over the carbonate, both perhaps depending on conversion into the chloride for absorption.

D. In Chart 4 are shown the serum calcium curves of thyro-parathyroprivic dogs after oral administration of calcium lactate, 1.5 gm. per kilo of body weight. The curves, in general, simulate those in which this salt has been given to normal dogs. The chief difference lies in a tendency toward slower return to the preingestive calcium value.

Dog XVII shows the difference in response between a dog in mild and very severe tetany. Under Curve XVIIa the effect of the lactate is shown on a dog with mild tetany, and under No. XVIIb the effect on the same dog with very severe tetany. In the latter case there is definitely delayed absorption, whereas in the former absorption is as rapid as in a normal animal. Perhaps during severe tetany congestion of the mucous membranes due to poor circulation interferes with absorption.

The evidence presented here together with that under Section B above emphasizes the necessity of administering calcium lactate in doses of at least 1.5 gm. per kilo of body weight in the control of tetany in dogs. Luckhardt's suggestion as cited above is therefore supported by proof of chemical as well as physiological nature.

The failure of some investigators to find an increase in the blood calcium after oral administration of certain calcium salts may be due to several factors, such as giving too small quantities, and drawing the blood for analyses when the calcium level may have returned to its preingestion value.

CONCLUSIONS.

1. Calcium salts such as the lactate, chloride, and glycerophosphate when administered orally in amounts containing a

calcium equivalent of 0.2727 gm. of the oxide per kilo of body weight are absorbed rapidly enough to increase definitely the serum calcium level in dogs, whereas the relatively insoluble salts such as the carbonate, in like quantity, are inconstant in this respect.

2. Calcium salts such as the lactate when ingested in quantities with a calcium equivalent of less than 0.2727 gm. of the oxide per kilo of body weight, simulate the less soluble salts in their effect on the serum calcium of dogs, for an increase is then inconstant.

3. Calcium lactate is preferable to the chloride even though it must be given in greater bulk; for it is less irritating to the gastric mucosa.

4. Calcium salts control tetany by increasing the blood calcium level of parathyroprivic dogs.

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INDEX TO AUTHORS

A

- Alsberg, C. L. See DILL and ALSBERG, 279
- Andrews, James C. The optical activity of cystine, 147
- . The oxidation of cystine, 161
- Atchley, Dana W., and Nichols, Emily G. The influence of protein concentration on the conductivity of human serum, 729

B

- Barkus, O. See MORGULIS and BARKUS, 1
- Baudisch, Oskar. See WELO and BAUDISCH, 215
- Bazin, Eleanor V. See RABINOWITCH, 55
- Becker, J. Ernestine. See MCCOLLUM, SIMMONDS, BECKER, and SHIPLEY, 97
- Burrell, Robin C., and Phillips, Thomas G. The determination of nitrate nitrogen in plants, 229

C

- Calvin, Dea B. See HENDRIX and CALVIN, 197
- Chambers, W. H., and Deuel, H. J., Jr. Animal calorimetry. Thirtieth paper.

The metabolism of glycerol in phlorhizin diabetes, 21

- Chambers, W. H. See DEUEL and CHAMBERS, 7
- Clark, Guy W. Acid- and base-forming elements in foods, 597
- Collins, Arnold M. See JACOBS and COLLINS, 491
- Cook, Donald H. Temperature coefficients of enzymic activity and the heat destruction of pancreatic and malt amylases, 135
- Cori, Carl F., and Cori, Gerty T. The carbohydrate metabolism of tumors. II. Changes in the sugar, lactic acid, and CO₂-combining power of blood passing through a tumor, 397
- Cori, Gerty T. See CORI and CORI, 397

D

- Denis, W., and Leche, Stella. On the distribution of injected sulfates in tissues, 565
- and — . A method for the determination of total sulfates in tissues, 561
- Deuel, H. J., Jr., and Chambers, W. H. The rate of elimination of ingested sugars in phlorhizin diabetes, 7

- Deuel, H. J., Jr. See CHAMBERS and DEUEL, 21
 Dill, D. B., and Alsberg, C. L. Preparation, solubility, and specific rotation of wheat gliadin, 279
 Doolittle, Dortha Bailey. See SMITH and DOOLITTLE, 665

E

- Eagles, Blythe A. See HUNTER and EAGLES, 623
 Elvehjem, C. A. See HART, STEENBOCK, ELVEHJEM, and WADDELL, 67

F

- Frith, Althea B. See RABINOWITCH, 55, 617

G

- Garlock, Bertha. See HELLER, McELROY, and GARLOCK, 255
 Gersdorff, Charles E. F. See JONES, MOELLER, and GERSDORFF, 59
 Goldberg, S. A. See MAYNARD, GOLDBERG, and MILLER, 643
 Gortner, Ross Aiken. See HOFFMAN and GORTNER, 371

H

- Haden, Russell L., and Orr, Thomas G. Chemical findings in the blood of the normal dog, 479
 Haller, H. L. See LEVENE and HALLER, 49
 Halpin, J. G. See HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON, 579

- Hamilton, Bengt. A comparison of the concentrations of inorganic substances in serum and spinal fluid, 101
 Harned, Ben K. The sugar content of blood, 555
 Hart, E. B., Steenbock, H., Elvehjem, C. A., and Waddell, J. Iron in nutrition. I. Nutritional anemia on whole milk diets and the utilization of inorganic iron in hemoglobin building, 67
 —, —, and Lepkovsky, S. Is the antirachitic factor of cod liver oil, when mixed with ground grains, destroyed through storage? 571
 —, —, Kletzien, S. W. F., Halpin, J. G., and Johnson, O. N. The nutritional requirement of the chicken. V. The influence of ultraviolet light on the production, hatchability, and fertility of the egg, 579
 Hastings, A. Baird, and Sendroy, Julius, Jr. The effect of variation in ionic strength on the apparent first and second dissociation constants of carbonic acid, 445
 —, —, and Robson, William. Studies of acidosis. XXI. The colorimetric determination of the pH of urine, 381
 —. See MURRAY and HASTINGS, 265
 —. See VAN SLYKE, HASTINGS, MURRAY, and SENDROY, 701
 Heller, V. G., McElroy, C. H., and Garlock, Bertha. The effect of the bacterial flora on the biological test for vitamin B, 255

- Henderson, L. J., and Murray, C. D.** Blood as a physico-chemical system. III. Deductions concerning the capillary exchange, 407
- Hendrix, Byron M., and Calvin, Dea B.** The loss of bases in diuresis and its effect upon the alkali reserve of the blood, 197
- Hjort, Axel M.** The influence of orally administered calcium salts on the serum calcium of normal and thyreoparathyroprivic dogs, 783
- , **Robison, S. C., and Tenedick, F. H.** An extract obtained from the external bovine parathyroid glands capable of inducing hypercalcemia in normal and thyreoparathyroprivic dogs, 117
- Hoffman, Walter F.** Sulfur in proteins. II. The effect of mild alkaline hydrolysis upon hair, 251
- and **Gortner, Ross Aiken.** The electrodialysis of agar. A method for the preparation of the free agar-acid, 371
- Holbøll, Svend Aage.** See LUNDSGAARD and HOLBØLL, 305, 323, 343, 363
- Hunter, George, and Eagles, Blythe A.** The isolation from blood of a hitherto unknown substance, and its bearing on present methods for the estimation of uric acid, 623

J

- Jacobs, Walter A., and Collins, Arnold M.** Strophanthin.

- VIII. The carbonyl group of strophanthidin, 491
- Johnson, O. N.** See HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON, 579
- Jones, D. Breese, Moeller, Otto, and Gersdorff, Charles E. F.** The nitrogen distribution and percentages of some amino acids in the muscle of the shrimp, *Peneus setiferus* (L.), 59

K

- Kletzien, S. W. F.** See HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON, 579

L

- Leche, Stella.** See DENIS and LECHE, 561, 565
- Lepkovsky, S.** See HART, STEENBOCK, and LEPKOVSKY, 571
- See HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON, 579
- Levene, P. A.** The mucoproteins of the snails, *Helix aspersa* and *Helix pomatia*, 683
- and **Haller, H. L.** The configurational relationships between β -hydroxy acids and α -hydroxy acids and between the latter and secondary alcohols, 49
- and **Meyer, G. M.** The numerical values of the optical rotation of methylated gluconic acids and of their salts, 535
- and **Mikeska, L. A.** On the oxidation of secondary

- mercaptans into corresponding sulfonic acids, 515
- Levene, P. A., and Mikeska, L. A.** Substitution by halogen of the hydroxyl in secondary alcohols, 507
- and **Rolf, Ida P.** Bromolecithins. I. Fractionation of brominated soy bean lecithins, 545
- and **Simms, H. S.** The dissociation constants of plant nucleotides and nucleosides and their relation to nucleic acid structure, 519
- and —. Lactone formation from mono- and dicarboxylic sugar acids, 31
- and **Sobotka, Harry.** Synthetic nucleosides. I. Theophylline pentosides, 463
- and —. II. Substituted uracil xylosides, 469
- and —. The thio-sugar from yeast, 551
- and **van der Hoeven, B. J. C.** The concentration of vitamin B. II, 483
- Lewis, Howard B.** The metabolism of sulfur. IX. The effect of repeated administration of small amounts of cystine, 187
- Liljestrand, S. H., and Wilson, D. Wright.** The excretion of lactic acid in the urine after muscular exercise, 773
- Long, W. L.** See **WILSON, LONG, THOMPSON, and THURLOW,** 755
- Lundsgaard, Christen, and Holbøll, Svend Aage.** Studies in carbohydrate metabolism. II. Investigations into the mutarotation of β -glucose under various conditions, 305
- Lundsgaard, Christen, and Holbøll, Svend Aage.** III. Investigations into the nature of the glucose in the blood of normal individuals, 323
- and —. IV. Investigations into the nature of the glucose in the blood of patients with diabetes mellitus and of patients with benign glycosuria, 343
- and —. V. Investigations into the form of glucose in different body fluids, 363
- ### M
- Maynard, L. A., Goldberg, S. A., and Miller, R. C.** The influence of sunlight on bone development in swine, 643
- McCollum, E. V., Simmonds, Nina, Becker, J. Ernestine, and Shipley, P. G.** Studies on experimental rickets. XXVI. A diet composed principally of purified food-stuffs for use with the "line test" for vitamin D studies, 97
- McElroy, C. H.** See **HELLER, McELROY, and GARLOCK,** 255
- Meyer, G. M.** See **LEVENE and MEYER,** 535
- Mikeska, L. A.** See **LEVENE and MIKESKA,** 507, 515
- Miller, R. C.** See **MAYNARD, GOLDBERG, and MILLER,** 643
- Moeller, Otto.** See **JONES, MOELLER, and GERSDORFF,** 59

Morgan, William O. P. See
MURRAY and MORGAN, 419

Morgulis, Sergius, and Barkus, O. Studies on glycolysis *in vitro*, 1

Murray, Cecil D., and Hastings, A. Baird. The maintenance of carbonic acid equilibrium in the body, with especial reference to the influence of respiration and kidney function on CO_2 , H^+ , HCO_3' , and CO_3'' concentrations in plasma, 265

— and **Morgan, William O. P.** Oxygen exchange, blood, and the circulation. A coordinated treatment of the factors involved in oxygen supply on the basis of the diffusion theory, 419

— See HENDERSON and MURRAY, 407

— See VAN SLYKE, HASTINGS, MURRAY, and SENDROY, 701

N

Nichols, Emily G. See ATCHLEY and NICHOLS, 729

O

Okey, Ruth, and Robb, Elda I. Studies of the metabolism of women. I. Variations in the fasting blood sugar level and in sugar tolerance in relation to the menstrual cycle, 165

Orr, Thomas G. See HADEN and ORR, 479

P

Phillips, Thomas G. See BURRELL and PHILLIPS, 229

R

Rabinowitch, I. M. Blood sugar time curves following the ingestion of dihydroxyacetone, 55

— Urea tests of renal efficiency. I, 617

Robb, Elda I. See OKEY and ROBB, 165

Robison, S. C. See HJORT, ROBISON, and TENDICK, 117

Robson, William. See HASTINGS, SENDROY, and ROBSON, 381

Rolf, Ida P. See LEVENE and ROLF, 545

Ross, Effie C. See STADIE and ROSS, 735

Rothwell, Carmen S. Direct precipitation of calcium in cows' milk, 129

S

Salmon, W. D. Vitamin B in the excreta of rats on a diet low in this factor, 457

Scott, D. A. A further investigation of the chemical properties of insulin, 601

Sendroy, Julius, Jr. See HASTINGS and SENDROY, 445

— See HASTINGS, SENDROY, and ROBSON, 381

— See VAN SLYKE, HASTINGS, MURRAY, and SENDROY, 701

Shipley, P. G. See MCCOLLUM, SIMMONDS, BECKER, and SHIPLEY, 97

Simmonds, Nina. See MCCOLLUM, SIMMONDS, BECKER, and SHIPLEY, 97

Simms, H. S. See LEVENE and SIMMS, 31, 519

Smith, H. Monmouth, and Doolittle, Dortha Bailey. Energy expenditure of women during horizontal walking at different speeds, 665

Sobotka, Harry. See LEVENE and SOBOTKA, 463, 469, 551

Stadie, William C., and Ross, Effie C. A micro method for the determination of base in blood and serum and other biological materials, 735

Steenbock, H. See HART, STEENBOCK, ELVEHJEM, and WADDELL, 67

— See HART, STEENBOCK, and LEPKOVSKY, 571

— See HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON, 579

Stern, Hans T. The colorimetric pH test of water or unbuffered solutions, 677

Sumner, James B. A more specific reagent for the determination of sugar in urine, 393

T

Tendick, F. H. See HJORT, ROBISON, and TENDICK, 117

Thompson, H. S. See WILSON, LONG, THOMPSON, and THURLOW, 755

Thurlow, Sylva. See WILSON, LONG, THOMPSON, and THURLOW, 755

U

Updegraff, Helen. See LEWIS, 187

V

van der Hoeven, B. J. C. See LEVENE and VAN DER HOEVEN, 483

Van Slyke, Donald D., Hastings, A. Baird, Murray, Cecil D., and Sendroy, Julius, Jr. Studies of gas and electrolyte equilibria in blood. VIII. The distribution of hydrogen, chloride, and bicarbonate ions in oxygenated and reduced blood, 701

Vickery, Hubert Bradford. Some nitrogenous constituents of the juice of the alfalfa plant. IV. The betaine fraction, 81

— VI. Asparagine and amino acids in alfalfa, 657

— and **Vinson, Carl G.** Some nitrogenous constituents of the juice of the alfalfa plant. V. The basic lead acetate precipitate, 91

Vinson, Carl G. See VICKERY and VINSON, 91

W

Waddell, J. See HART, STEENBOCK, ELVEHJEM, and WADDELL, 67

Welo, Lars A., and Baudisch, Oskar. On the catalytically active and inactive forms of ferric oxide, 215

Wesson, Laurence G. On a possible relationship of arachidonic acid to the saturated fatty acids in fatty acid metabolism, 235

Wilson, D. Wright, Long, W. L., Thompson, H. C., and Thurlow, Sylva. Changes in the composition of the urine after muscular exercise, 755

— See LILJESTRAND and WILSON, 773

INDEX TO SUBJECTS.

A

- Acetate:**
 Lead, basic, precipitated
 from alfalfa (VICKERY
 and VINSON) 91
- Acid(s):**
 -forming elements in foods
 (CLARK) 597
- Acidosis:**
 XXI (HASTINGS, SENDROY,
 and ROBSON) 381
- Agar-acid:**
 Free, method for prepara-
 tion (HOFFMAN and
 GORTNER) 371
- Agar-agar:**
 Electrodialysis (HOFFMAN
 and GORTNER) 371
- Alcohol:**
 Secondary, α -hydroxy acids
 and, β -hydroxy acids and
 α -hydroxy acids, config-
 urational relation (LE-
 VENE and HALLER) 49
- , hydroxyl, substitution
 by halogen (LEVENE
 and MIKESKA) 507
- Alfalfa:**
 Amino acid (VICKERY) 657
- Asparagine (VICKERY) 657
- Juice, nitrogenous consti-
 tuents. IV (VICKERY) 81
- , — —. V (VICKERY
 and VINSON) 91
- , — —. VI (VICKERY) 657

Alkali:

- Hydrolysis, mild, on hair
 (HOFFMAN) 251
- Reserve, blood, effect of
 loss of bases in diuresis
 (HENDRIX and CALVIN) 197

Amino acid(s):

- Alfalfa (VICKERY) 657
- Peneus setiferus*, muscle,
 percentages (JONES,
 MOELLER, and GERS-
 DORFF) 59
- Shrimp, muscle, percent-
 ages (JONES, MOELLER,
 and GERSDORFF) 59

Amylase:

- Malt, heat destruction
 (COOK) 135
- Pancreas, heat destruction
 (COOK) 135

Anemia:

- Nutritional, on whole milk
 diets (HART, STEEN-
 BOCK, ELVEHJEM, and
 WADDELL) 67

Arachidonic acid:

- Metabolism, fatty acid,
 saturated fatty acids in,
 relation (WESSON) 235

Asparagine:

- Alfalfa (VICKERY) 657

Aspersa:

- Helix*, mucoproteins (LE-
 VENE) 683

B**Bacterium:**

- Flora, bacterial, effect on
biological test for vitamin
B (HELLER, McELROY,
and GARLOCK) 255

Base:

- Biological material, micro
method, determination
(STADIE AND ROSS) 735

- Blood, micro method, de-
termination (STADIE and
ROSS) 735

- Diuresis, loss, effect on
alkali reserve of blood
(HENDRIX and CALVIN) 197

- forming elements in foods
(CLARK) 597

- Lead acetate, precipitated
from alfalfa (VICKERY
and VINSON) 91

- Serum, micro method, de-
termination (STADIE and
ROSS) 735

Betaine:

- Alfalfa (VICKERY) 81

Bicarbonate:

- Concentration in plasma,
influence of kidney func-
tion (MURRAY and
HASTINGS) 265

- — —, influence of res-
piration (MURRAY and
HASTINGS) 265

- Ion, in blood, oxygenated,
distribution (VAN SLYKE,
HASTINGS, MURRAY, and
SENDROY) 701

- , — —, reduced, distri-
bution (VAN SLYKE,
HASTINGS, MURRAY, and
SENDROY) 701

Biology:

- Base, in biological ma-
terials, micro method, de-
termination (STADIE and
ROSS) 735

- Vitamin B, biological test
for, effect of bacterial
flora (HELLER, Mc-
ELROY, and GARLOCK) 255

Blood:

- Alkali reserve, effect of
loss of bases in diuresis
(HENDRIX and CALVIN) 197

- Base, micro method, deter-
mination (STADIE and
ROSS) 735

- Carbon dioxide-combining
power, changes while
passing through tumor
(CORI and CORI) 397

- Chemical findings, of nor-
mal dog (HADEN and
ORR) 479

- Equilibrium, electrolyte.
VIII (VAN SLYKE, HAS-
TINGS, MURRAY, and
SENDROY) 701

- , gas. VIII (VAN
SLYKE, HASTINGS, MUR-
RAY, and SENDROY) 701

- Glucose, of normal individ-
uals (LUNDGAARD and
HOLBØLL) 323

- , — patients with benign
glycosuria (LUNDGAARD
and HOLBØLL) 343

- , — — diabetes melli-
tus (LUNDGAARD and
HOLBØLL) 343

- Isolation of unknown sub-
stance from, and its
bearing on present

Blood—continued.

- methods for estimation of uric acid (HUNTER and EAGLES) 623
- Lactic acid, changes while passing through tumor (CORI and CORI) 397
- Oxygenation, bicarbonate ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701
- , chloride ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701
- , hydrogen ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701
- Oxygen exchange, circulation and, treatment on basis of diffusion theory (MURRAY and MORGAN) 419
- Physicochemical system. III (HENDERSON and MURRAY) 407
- Reduction, bicarbonate ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701
- , chloride ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701
- , hydrogen ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701
- Sugar, changes while passing through tumor (CORI and CORI) 397
- content (HARNED) 555
- level, variations in fasting, relation to mens-

Blood—continued.

- trual cycle (OKEY and ROBB) 165
- time curve following ingestion of dihydroxyacetone (RABINOWITCH) 55

Body:

- Equilibrium, carbonic acid (MURRAY and HASTINGS) 265

Bone:

- Swine, influence of sunlight on development (MAYNARD, GOLDBERG, and MILLER) 643

Bromolecithin:

- I (LEVENE and ROLF) 545
- Soy bean, fractionation (LEVENE and ROLF) 545

Buffer:

- Solution, unbuffered, colorimetric hydrogen ion concentration test (STERN) 677

C**Calcium:**

- Precipitation, in cows' milk (ROTHWELL) 129
- Serum, normal dogs, influence of orally administered calcium salts (HJORT) 783
- , thyreoparathyroprivic dogs, influence of orally administered calcium salts (HJORT) 783

Calcium salt(s):

- Orally administered, serum calcium of normal dogs, influence on, of (HJORT) 783
- — — of thyreoparathyroprivic dogs, influence on, of (HJORT) 783

Calorimetry:

Animal. XXX (CHAMBERS and DEUEL)

21

Capillary:

Exchange, deductions concerning (HENDERSON and MURRAY)

407

Carbohydrate:

Metabolism. II (LUNDSSGAARD and HOLBØLL)

305

—, III (LUNDSSGAARD and HOLBØLL)

323

—, IV (LUNDSSGAARD and HOLBØLL)

343

—, V (LUNDSSGAARD and HOLBØLL)

363

—, of tumors. II (CORI and CORI)

397

Carbonate:

Concentration in plasma, influence of kidney function (MURRAY and HASTINGS)

265

— — —, influence of respiration (MURRAY and HASTINGS)

265

Carbon dioxide:

—combining power of blood, changes while passing through tumor (CORI and CORI)

397

Concentration in plasma, influence of kidney function (MURRAY and HASTINGS)

265

— — —, influence of respiration (MURRAY and HASTINGS)

265

Carbonic acid:

Dissociation constant, first, effect of variation in ionic strength (HASTINGS and SENDROY)

445

— — —, second, effect of variation in ionic

Carbonic acid—continued.

strength (HASTINGS and SENDROY)

445

Equilibrium in body (MURRAY and HASTINGS)

265

Carbonyl:

Strophanthidin (JACOBS and COLLINS)

491

Catalysis:

Ferric oxide, active forms (WELO and BAUDISCH)

215

— — —, inactive forms (WELO and BAUDISCH)

215

Cattle:

Parathyroid, extract, hypercalcemia in normal dogs induced by (HJORT, ROBISON, and TENDICK)

117

— — — — —, thyreoparathyroprivic dogs induced by (HJORT, ROBISON, and TENDICK)

117

Chicken:

Nutrition, requirement. V (HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON)

579

Chloride:

Ion, in blood, oxygenated, distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY)

701

— — — — —, reduced, distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY)

701

Circulation:

Oxygen exchange, blood and, treatment on basis of diffusion theory (MURRAY and MORGAN)

419

Cod liver oil:

Antirachitic factor, is it destroyed through storage when mixed with ground grains (HART, STEENBOCK, and LEPKOVSKY) 571

Coefficient:

Temperature, of enzymic activity (COOK) 135

Colorimetry:

Hydrogen ion concentration test of unbuffered solutions (STERN) 677

— — — — of water (STERN) 677

Urine, hydrogen ion, determination (HASTINGS, SENDROY, and ROBSON) 381

Conductivity:

Serum, human, protein concentration on (ATCHLEY and NICHOLS) 729

Configuration:

β -Hydroxy and α -hydroxy acids and α -hydroxy acids and secondary alcohols, relation (LEVENE and HALLER) 49

Cystine:

Optical activity (ANDREWS) 147

Oxidation (ANDREWS) 161

Sulfur, metabolism, effect of small amounts (LEWIS) 187

D**Diabetes:**

Mellitus, glucose in blood of patients (LUNDGAARD and HOLBØLL) 343

Diabetes—continued.

Phlorhizin, elimination of ingested sugars (DEUEL and CHAMBERS) 7

—, metabolism of glycerol (CHAMBERS and DEUEL) 21

Diet:

Purified foodstuffs, in line test for vitamin D (MCCOLLUM, SIMMONDS, BECKER, and SHIPLEY) 97

Vitamin B, low, vitamin B in excreta of rats (SALMON) 457

Whole milk, nutritional anemia (HART, STEENBOCK, ELVEHJEM, and WADDELL) 67

Diffusion:

Theory, oxygen supply, treatment on basis (MURRAY and MORGAN) 419

Dihydroxyacetone:

Blood sugar time curve following ingestion (RABINOWITCH) 55

Dissociation constant:

First, carbonic acid, effect of variation in ionic strength (HASTINGS and SENDROY) 445

Nucleoside, plant, nucleic acid structure, relation (LEVENE and SIMMS) 519

Nucleotide, plant, nucleic acid structure, relation (LEVENE and SIMMS) 519

Second, carbonic acid, effect of variation in ionic strength (HASTINGS and SENDROY) 445

Diuresis:

Base, loss, effect on alkali
reserve of blood (HENDRIX and CALVIN)

197

E**Efficiency:**

Kidney, urea tests. I
(RABINOWITCH)

617

Egg:

Fertility, influence of ultra-violet light (HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON)

579

Hatchability, influence of ultra-violet light (HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON)

579

Production, influence of ultra-violet light (HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON)

579

Electrodialysis:

Agar-agar (HOFFMAN and GORTNER)

371

Electrolyte:

Equilibrium, in blood. VIII (VAN SLYKE, HASTINGS, MURRAY, and SENDROY)

701

Elimination:

Sugar, ingested, in phlo-rhizin diabetes (DEUEL and CHAMBERS)

7

Energy:

Expenditure of women during horizontal walking at different speeds (SMITH and DOOLITTLE)

665

Enzyme:

Temperature coefficients of enzymic activity (COOK)

135

Equilibrium:

Carbonic acid, in body (MURRAY and HASTINGS)

265

Electrolyte, in blood. VIII (VAN SLYKE, HASTINGS, MURRAY, and SENDROY)

701

Gas, in blood. VIII (VAN SLYKE, HASTINGS, MURRAY, and SENDROY)

701

Excretion:

Lactic acid, in urine, after muscular exercise (LILJESTRAND and WILSON)

773

Vitamin B, in excreta of rats, diet low in vitamin B (SALMON)

457

Exercise:

Muscle, changes in urine, composition after (WILSON, LONG, THOMPSON, and THURLOW)

755

Extract:

Parathyroid, bovine, hypercalcemia in normal dogs induced by (HJORT, ROBISON, and TENDICK)

117

—, —, — — thyreoparathyroprivic dogs induced by (HJORT, ROBISON, and TENDICK)

117

F**Fasting:**

Blood sugar level, variations, relation to menstrual cycle (OKEY and ROBB)

165

Fatty acid:

Metabolism, arachidonic acid and saturated fatty acids in, relation (WESSON)

235

Ferric oxide:Catalysis, active forms
(WELO and BAUDISCH)

215

—, inactive forms (WELO
and BAUDISCH)

215

Fertilization:Egg, influence of ultra-
violet light (HART,
STEENBOCK, LEPKOVSKY,
KLETZIEN, HALPIN, and
JOHNSON)

579

Fluid:Spinal, inorganic sub-
stances in serum and,
comparison of concen-
trations (HAMILTON)

101

Food:Acid-forming elements
(CLARK)

597

Base-forming elements
(CLARK)

597

Purified, diet, in line test
for vitamin D (McCOL-
LUM, SIMMONDS, BECKER,
and SHIPLEY)

97

G**Gas:**Equilibrium, in blood.
VIII (VAN SLYKE, HAS-
TINGS, MURRAY, and
SENDROY)

701

Gland:Parathyroid. *See* Para-
thyroid.**Gliadin:**Wheat, preparation (DILL
and ALSBERG)

279

—, solubility (DILL and
ALSBERG)

279

—, specific rotation (DILL
and ALSBERG)

279

Gluconic acid(s):

Methylated, and their

Gluconic acid(s)—continued.salts, optical rotation
(LEVENE and MEYER)

535

Glucose:Blood, of normal individ-
uals (LUNDGAARD and
HOLBØLL)

323

—, — patients with benign
glycosuria (LUNDGAARD
and HOLBØLL)

343

—, — — — diabetes melli-
tus (LUNDGAARD and
HOLBØLL)

343

Body fluid (LUNDGAARD
and HOLBØLL)

363

 β -Glucose:Mutarotation (LUND-
GAARD and HOLBØLL)

305

Glycerol:Metabolism, in phlorhizin
diabetes (CHAMBERS and
DEUEL)

21

Glycolysis:*In vitro* (MORGULIS and
BARKUS)

1

Glycosuria:Benign, glucose in blood of
patients (LUNDGAARD
and HOLBØLL)

343

Grain:Cod liver oil, antirachitic
factor, is it destroyed
through storage when
mixed with ground
grains (HART, STEEN-
BOCK, and LEPKOVSKY)

571

H**Hair:**Hydrolysis, mild alkaline
(HOFFMAN)

251

Halogen:Hydroxyl, in secondary al-
cohols, substitution by

Halogen—continued.(LEVENE and MIKESKA)
507**Hatchability:**Egg, influence of ultra-
violet light (HART,
STEENBOCK, LEPKOVSKY,
KLETZIEN, HALPIN, and
JOHNSON) 579**Heat:**Amylase, malt, destruction
(COOK) 135
—, pancreatic, destruction
(COOK) 135**Helix:***aspersa*, mucoproteins (LE-
VENE) 683
pomatia, mucoproteins
(LEVENE) 683**Hemoglobin:**Inorganic iron, utilization
in building (HART,
STEENBOCK, ELVEHJEM,
and WADDELL) 67**Hydrogen:**Ion concentration in
plasma, influence of kid-
ney function (MURRAY
and HASTINGS) 265
— — — —, influence of
respiration (MURRAY
and HASTINGS) 265
— — test, colorimetric, of
unbuffered solutions
(STERN) 677
— — —, —, of water
(STERN) 677
—, in blood, oxygenated,
distribution (VAN
SLYKE, HASTINGS, MUR-
RAY, and SENDROY) 701
—, — —, reduced, distri-
bution (VAN SLYKE,
HASTINGS, MURRAY, and
SENDROY) 701**Hydrogen—continued.**Ion, of urine, colorimetric
determination (HAS-
TINGS, SENDROY, and
ROBSON) 381**Hydrolysis:**Alkaline, mild, on hair
(HOFFMAN) 251 **α -Hydroxy acid(s):** β -Hydroxy acids and, α -
hydroxy acids and
secondary alcohols, con-
figurational relation (LE-
VENE and HALLER) 49 **β -Hydroxy acid(s):** α -Hydroxy acids and, α -
hydroxy acids and
secondary alcohols, con-
figurational relation
(LEVENE and HALLER) 49**Hydroxyl:**Alcohol, secondary, sub-
stitution by halogen
(LEVENE and MIKESKA)
507**Hypercalcemia:**Dog, normal, induced by
extract from bovine
parathyroid (HJORT,
ROBISON, and TEN-
DICK) 117
—, thyreoparathyroprivic,
induced by extract from
bovine parathyroid
(HJORT, ROBISON, and
TENDICK) 117**I****Inorganic:**Iron, utilization in hemo-
globin building (HART,
STEENBOCK, ELVEHJEM,
and WADDELL) 67**Insulin:**Chemical properties
(SCOTT) 601

In vitro:

Glycolysis (MORGULIS and BARKUS) 1

Ion:

Bicarbonate, in blood, oxygenated, distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701

—, —, reduced, distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701

Carbonic acid, first dissociation constant, effect of variation in ionic strength (HASTINGS and SENDROY) 445

—, —, second dissociation constant, effect of variation in ionic strength (HASTINGS and SENDROY) 445

Chloride, in blood, oxygenated, distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701

—, —, reduced, distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701

Hydrogen, concentration in plasma, influence of kidney function (MURRAY and HASTINGS) 265

—, —, —, influence of respiration (MURRAY and HASTINGS) 265

—, — test, colorimetric, of unbuffered solutions (STERN) 677

—, —, —, of water (STERN) 677

—, in blood, oxygenated, distribution (VAN SLYKE,

Ion—continued.

HASTINGS, MURRAY, and SENDROY) 701

—, —, reduced, distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701

—, of urine, colorimetric determination (HASTINGS, SENDROY, and ROBSON) 381

Iron:

Inorganic, utilization in hemoglobin building (HART, STEENBOCK, ELVEHJEM, and WADDELL) 67

Nutrition. I (HART, STEENBOCK, ELVEHJEM, and WADDELL) 67

J**Juice:**

Alfalfa, nitrogenous constituents. IV (VICKERY) 81

—, —, —. V (VICKERY and VINSON) 91

—, —, —. VI (VICKERY) 657

K**Kidney:**

Concentration in plasma, bicarbonate, influence on, of (MURRAY and HASTINGS) 265

—, —, —, carbonate, influence on, of (MURRAY and HASTINGS) 265

—, —, —, carbon dioxide, influence on, of (MURRAY and HASTINGS) 265

—, —, —, hydrogen ion, influence on, of (MURRAY and HASTINGS) 265

Kidney—continued.

Urea tests of efficiency. I
(RABINOWITCH)

617

L**Lactic acid:**

Blood, changes while passing through tumor
(CORI and CORI)

397

Excretion, in urine, after muscular exercise (LILJESTRAND and WILSON)

773

Lactone:

Formation from dicarboxylic sugar acids (LEVENE and SIMMS)

31

— — monocarboxylic sugar acids (LEVENE and SIMMS)

31

Lead:

Acetate, basic, precipitated from alfalfa (VICKERY and VINSON)

91

Light:

Ultra-violet, egg fertility (HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON)

579

—, — hatchability (HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON)

579

—, — production (HART, STEENBOCK, LEPKOVSKY, KLETZIEN, HALPIN, and JOHNSON)

579

M**Malt:**

Amylase, heat destruction (COOK)

135

Menstruation:

Blood sugar level, variations in fasting, relation (OKEY and ROBB)

165

Sugar tolerance, variations, relation (OKEY and ROBB)

165

Mercaptan:

Secondary, oxidation, into corresponding sulfonic acids (LEVENE and MIKESKA)

515

Metabolism:

Carbohydrate. II (LUNDSTAD and HOLBØLL)

305

—, III (LUNDSTAD and HOLBØLL)

323

—, IV (LUNDSTAD and HOLBØLL)

343

—, V (LUNDSTAD and HOLBØLL)

363

—, of tumors. II (CORI and CORI)

397

Fatty acid, arachidonic acid and saturated fatty acids in, relation (WESSON)

235

Glycerol, in phlorhizin diabetes (CHAMBERS and DEUEL)

21

Sulfur. IX (LEWIS)

187

Women. I (OKEY and ROBB)

165

Methylate:

Gluconic acid, methylated, and their salts, optical rotation (LEVENE and MEYER)

535

Milk:

Cow, calcium precipitation in (ROTHWELL)

129

Whole, nutritional anemia on diets (HART, STEEN-

Milk—continued.

BOCK, ELVEHJEM, and
WADDELL) 67

Mucoprotein:

Helix aspersa (LEVENE) 683

— *pomatia* (LEVENE) 683

Snail (LEVENE) 683

Muscle:

Exercise, changes in urine,
composition after (WIL-
SON, LONG, THOMPSON,
and THURLOW) 755

—, lactic acid excretion in
urine after (LILJESTRAND
and WILSON) 773

Peneus setiferus, amino acid
percentages (JONES,
MOELLER, and GERS-
DORFF) 59

— —, nitrogen distribu-
tion (JONES, MOELLER,
and GERSDORFF) 59

Shrimp, amino acid per-
centages (JONES, MOEL-
LER, and GERSDORFF) 59

—, nitrogen distribution
(JONES, MOELLER, and
GERSDORFF) 59

Mutarotation:

β -Glucose (LUNDSGAARD and
HOLBØLL) 305

N**Nitrate:**

Nitrogen, in plant (BUR-
RELL and PHILLIPS) 229

Nitrogen:

Alfalfa juice, nitrogenous
constituents. VI (VICK-
ERY) 657

Muscle, of *Peneus setiferus*,
distribution (JONES,

Nitrogen—continued.

MOELLER, and GERS-
DORFF) 59

—, — shrimp, distribution
(JONES, MOELLER, and
GERSDORFF) 59

Nitrate, in plant (BURRELL
and PHILLIPS) 229

Nitrogenous:

Constituent, alfalfa juice.
IV (VICKERY) 81

—, — —. V (VICKERY
and VINSON) 91

—, — —. VI (VICKERY)
657

Nucleic acid:

Plant nucleoside, dissocia-
tion constant, relation
(LEVENE and SIMMS) 519

— nucleotide, dissociation
constant, relation (LE-
VENE and SIMMS) 519

Nucleoside:

Plant, dissociation con-
stant, nucleic acid
structure, relation (LE-
VENE and SIMMS) 519

Synthesis. I (LEVENE and
SOBOTKA) 463

— II (LEVENE and
SOBOTKA) 469

Nucleotide:

Plant, dissociation con-
stant, nucleic acid struc-
ture, relation (LEVENE
and SIMMS) 519

Nutrition:

Anemia, on whole milk
diets (HART, STEEN-
BOCK, ELVEHJEM, and
WADDELL) 67

Chick, requirement. V
(HART, STEENBOCK,
LEPKOVSKY, KLETZIEN,
HALPIN, and JOHNSON)
579

Nutrition—continued.

- Iron in. I (HART, STEEN-
BOCK, ELVEHJEM, and
WADDELL) 67

O**Oxidation:**

- Cystine (ANDREWS) 161

- Mercaptan, secondary, into
corresponding sulfonic
acids (LEVENE and MI-
KESKA) 515

Oxygen:

- Exchange, blood and cir-
culation, treatment on
basis of diffusion theory
(MURRAY and MORGAN) 419

Oxygenation:

- Blood, bicarbonate ion dis-
tribution (VAN SLYKE,
HASTINGS, MURRAY, and
SENDROY) 701
—, chloride ion distribu-
tion (VAN SLYKE, HAS-
TINGS, MURRAY, and
SENDROY) 701
—, hydrogen ion distribu-
tion (VAN SLYKE, HAS-
TINGS, MURRAY, and
SENDROY) 701

P**Pancreas:**

- Amylase, heat destruction
(COOK) 135

Parathyroid:

- Cattle, extract, hypercal-
cemia in normal dogs
induced by (HJORT,
ROBISON, and TEN-
DICK) 117
—, —, — — thyreopara-
thyroprivic dogs induced
by (HJORT, ROBISON,
and TENDICK) 117

Peneus:

- setiferus*, muscle, amino
acid percentages (JONES,
MOELLER, and GERS-
DORFF) 59
—, —, nitrogen distribu-
tion (JONES, MOELLER,
and GERSDORFF) 59

Pentoside:

- Theophylline (LEVENE and
SOBOTKA) 463

Phlorhizin:

- Diabetes, elimination of
ingested sugars (DEUEL
and CHAMBERS) 7
—, metabolism of glycerol
(CHAMBERS and DEUEL) 21

Plant:

- Nitrogen, nitrate (BUR-
RELL and PHILLIPS) 229
Nucleoside, dissociation
constant, nucleic acid
structure, relation (LE-
VENE and SIMMS) 519
Nucleotide, dissociation
constant, nucleic acid
structure, relation (LE-
VENE and SIMMS) 519

Plasma:

- Bicarbonate concentration,
influence of kidney func-
tion (MURRAY and HAS-
TINGS) 265
—, —, — — respiration
(MURRAY and HAS-
TINGS) 265
Carbonate concentration,
influence of kidney func-
tion (MURRAY and HAS-
TINGS) 265
—, —, — — respiration
(MURRAY and HAS-
TINGS) 265

Plasma—continued.

- Carbon dioxide concentration, influence of kidney function (MURRAY and HASTINGS) 265
 — — —, influence of respiration (MURRAY and HASTINGS) 265
 Hydrogen ion concentration, influence of kidney function (MURRAY and HASTINGS) 265
 — — —, influence of respiration (MURRAY and HASTINGS) 265

Pomatia:

- Helix*, mucoproteins (LE-
 VENE) 683

Precipitation:

- Acetate, basic lead, from alfalfa (VICKERY and VINSON) 91
 Calcium, in cows' milk (ROTHWELL) 129

Protein:

- Concentration, human serum, conductivity (ATCHLEY and NICHOLS) 729
 Sulfur. II (HOFFMAN) 251

R**Rachitis:**

- Cod liver oil, antirachitic factor, is it destroyed through storage when mixed with ground grains (HART, STEENBOCK, and LEPKOVSKY) 571

Reduction:

- Blood, bicarbonate ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701
 —, chloride ion distribution (VAN SLYKE, HAS-

Reduction—continued.

- TINGS, MURRAY, and SENDROY) 701
 —, hydrogen ion distribution (VAN SLYKE, HASTINGS, MURRAY, and SENDROY) 701

Renal:

See Kidney.

Respiration:

- Concentration in plasma, bicarbonate influence on, of (MURRAY and HASTINGS) 265
 — — —, carbonate, influence on, of (MURRAY and HASTINGS) 265
 — — —, carbon dioxide, influence on, of (MURRAY and HASTINGS) 265
 — — —, hydrogen ion, influence on, of (MURRAY and HASTINGS) 265

Rickets:

- XXVI (McCOLLUM, SIMMONDS, BECKER, and SHIPLEY) 97

S**Salt(s):**

- Calcium, orally administered, serum calcium of normal dogs, influence on, of (HJORT) 783
 —, — —, serum calcium of thyreoparathyroprivic dogs, influence on, of (HJORT) 783
 Gluconic acid, methylated, and their salts, optical rotation (LEVENE and MEYER) 535

Serum:

- Base, micro method, de-

Serum—*continued*.

- termination (STADIE and ROSS) 735
- Calcium, normal dogs, influence of calcium salts orally administered (HJORT) 783
- , thyreoparathyroprivic dogs, influence of calcium salts orally administered (HJORT) 783

- Human, conductivity, protein concentration on (ATCHLEY and NICHOLS) 729

- Inorganic substance, and spinal fluid, comparison of concentrations (HAM-ILTON) 101

Setiferus:

- Peneus*, muscle, amino acid percentages (JONES, MOELLER, and GERSDORFF) 59
- , —, nitrogen distribution (JONES, MOELLER, and GERSDORFF) 59

Shrimp:

- Muscle, amino acid percentages (JONES, MOELLER, and GERSDORFF) 59
- , nitrogen distribution (JONES, MOELLER, and GERSDORFF) 59

Snail:

- Mucoprotein (LEVENE) 683

Soy bean:

- Bromolecithin, fractionation (LEVENE and ROLF) 545

Spinal fluid:

- See* Fluid.

Strophanthidin:

- Carbonyl group (JACOBS and COLLINS) 491

Strophanthin:

- VIII (JACOBS and COLLINS) 491

Sugar:

- Acid, dicarboxylic, lactone formation (LEVENE and SIMMS) 31
- , monocarboxylic, lactone formation (LEVENE and SIMMS) 31
- Blood, changes while passing through tumor (CORI and CORI) 397
- , content (HARNED) 555

- , level, variations in fasting, relation to menstrual cycle (OKEY and ROBB) 165

- , time curve following ingestion of dihydroxyacetone (RABINOWITCH) 55

- Diabetes, phlorhizin, elimination of ingested (DEUEL and CHAMBERS) 7

- Tolerance, variations, relation to menstrual cycle (OKEY and ROBB) 165

- Urine, specific reagent for determination (SUMNER) 393

Sulfate:

- Tissue, determination (DENIS and LECHE) 561

- , distribution of injected (DENIS and LECHE) 565

Sulfonic acid(s):

- Mercaptan, secondary, oxi-

Sulfonic acid(s)—*continued*.

dation, into corresponding (LEVENE and MIKESKA) 515

Sulfur:

Metabolism. IX (LEWIS) 187
Protein. II (HOFFMAN) 251

Sunlight:

Swine, bone, influence on development (MAYNARD, GOLDBERG, and MILLER) 643

Swine:

Bone development, influence of sunlight (MAYNARD, GOLDBERG, and MILLER) 643

T**Temperature:**

Coefficient, of enzymic activity (COOK) 135

Theophylline:

Pentoside (LEVENE and SOBOTKA) 463

Thio-sugar:

Yeast (LEVENE and SOBOTKA) 551

Thyreoparathyroprivic:

Dog, hypercalcemia, induced by extract from bovine parathyroid (HJORT, ROBISON, and TENDICK) 117

—, serum calcium, influence of orally administered calcium salts (HJORT) 783

Tissue:

Sulfate, determination (DENIS and LECHE) 561

—, injected, distribution (DENIS and LECHE) 565

Tumor:

Blood, carbon dioxide-combining power, changes while passing through (CORI and CORI) 397

—, lactic acid, changes while passing through (CORI and CORI) 397

—, sugar, changes while passing through (CORI and CORI) 397

Carbohydrate metabolism. II (CORI and CORI) 397

U**Uracil:**

Xyloside, substituted (LEVENE and SOBOTKA) 469

Urea:

Kidney efficiency test. I (RABINOWITCH) 617

Uric acid:

Blood, isolation of unknown substance from, and its bearing on present methods of estimating (HUNTER and EAGLES) 623

Urine:

Exercise, muscular, changes in composition after (WILSON, LONG, THOMPSON, and THURLOW) 755

Hydrogen ion, colorimetric determination (HASTINGS, SENDROY, and ROBSON) 381

Lactic acid, excretion, after muscular exercise (LILJESTRAND and WILSON) 773

Urine—*continued.*

Sugar, specific reagent for
determination (SUMNER) 393

V**Vitamin(s):**

B, biological test for, effect
of bacterial flora (HEL-
LER, McELROY, and
GARLOCK) 255

—, concentration. II
(LEVENE and VAN DER
HOEVEN) 483

—, in excreta of rats, diet
low in vitamin B (SAL-
MON) 457

D, diet of purified food-
stuffs in line test for
(McCOLLUM, SIMMONDS,
BECKER, and SHIPLEY) 97

W**Water:**

Hydrogen ion concentra-
tion test, colorimetric
(STERN) 677

Wheat:

Gliadin, preparation (DILL
and ALSBERG) 279

—, solubility (DILL and
ALSBERG) 279

—, specific rotation (DILL
and ALSBERG) 279

X**Xyloside:**

Uracil, substituted (LE-
VENE and SOBOTKA) 469

Y**Yeast:**

Thio-sugar (LEVENE and
SOBOTKA) 551

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BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
HENRY D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

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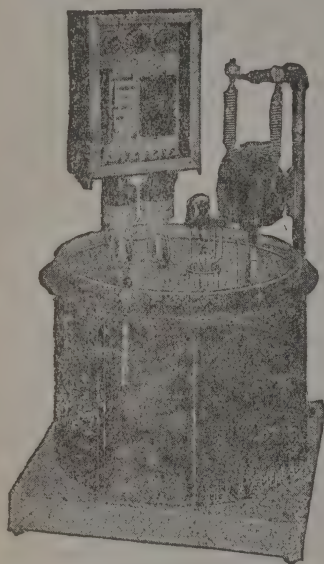
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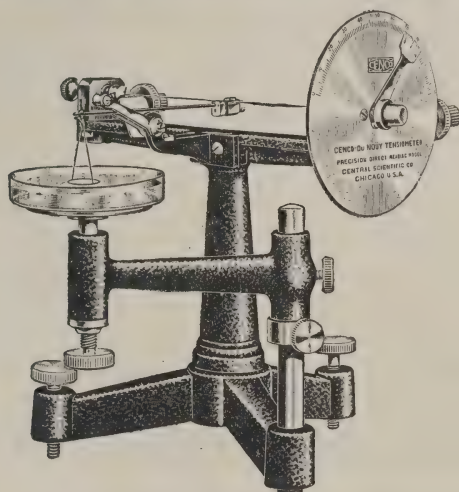
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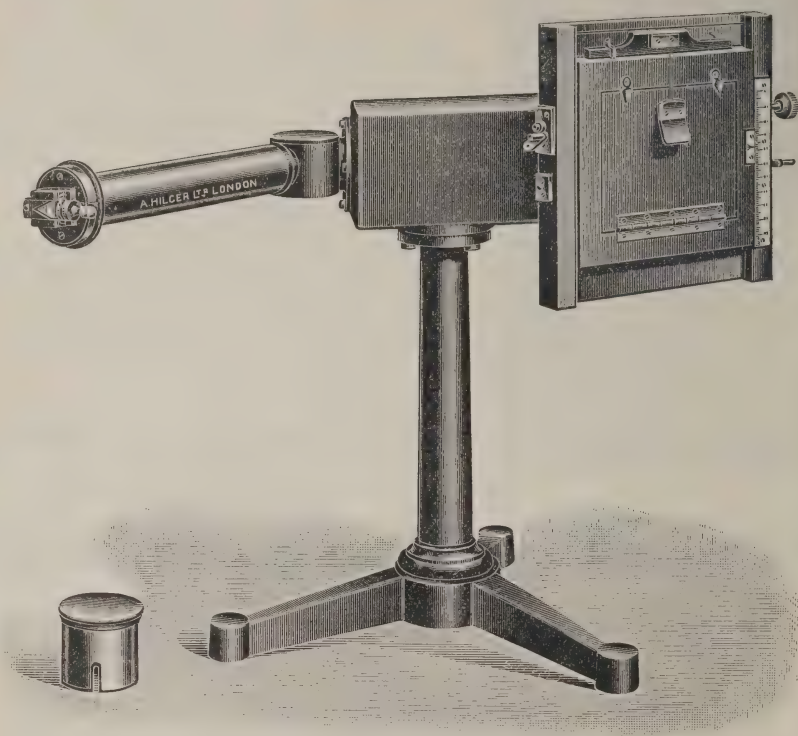
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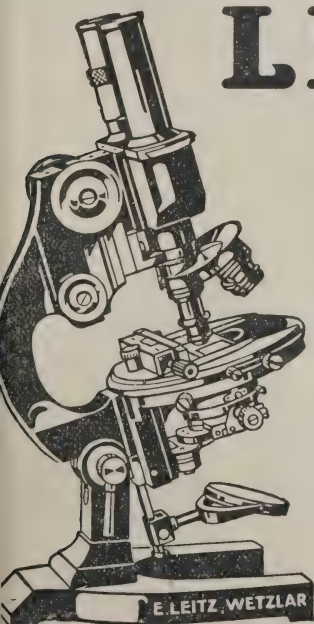
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CONTENTS OF VOL. IX, No. 2, OCTOBER 20, 1925

- MURRAY, HENRY A., JR. Physiological ontogeny. A. Chicken embryos. II. Catabolism. Chemical changes in fertile eggs during incubation. Selection of standard conditions.
- MURRAY, HENRY A., JR. Physiological ontogeny. A. Chicken embryos. III. Weight and growth rate as functions of age.
- CROZIER, W. J., and STIER, T. B. Temperature characteristic for locomotor activity in tent caterpillars.
- KUSHNER, IRVING. The effect of x-rays on the irritability of muscles in the frog.
- HAMMETT, FREDERICK S. A comparison of bone growth in length with bone growth in weight.
- MUDD, STUART. Electroendosmosis through mammalian serous membranes. II. Comparison of hydrogen ion reversal points with acetate and with citrate-phosphate buffers.
- NORTHROP, JOHN H. The influence of the intensity of light on the rate of growth and duration of life of *Drosophila*.
- CLARK, HARRY, and NORTHROP, JOHN H. The inactivation of trypsin by x-rays.
- HITCHCOCK, DAVID I. Some consequences of the theory of membrane equilibria.
- BAAS-BECKING, LOURENS G. M., and ROSS, P. A. Notes on microspectra. I. The absorption spectrum of *Euglena*.
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CONTENTS

HARNED, BEN K. The sugar content of blood.....	555
DENIS, W., AND LECHE, STELLA. A method for the determination of total sulfates in tissues.....	561
DENIS, W., AND LECHE, STELLA. On the distribution of injected sulfates in tissues....	565
HART, E. B., STEENBOCK, H., AND LEPKOVSKY, S. Is the antirachitic factor of cod liver oil, when mixed with ground grains, destroyed through storage? Plate 1.....	571
HART, E. B., STEENBOCK, H., LEPKOVSKY, S., KLETZIEN, S. W. F., HALPIN, J. G., AND JOHNSON, O. N. The nutritional requirement of the chicken. V. The influence of ultra-violet light on the production, hatchability, and fertility of the egg.....	579
CLARK, GUY W. Acid- and base-forming elements in foods.....	597
SCOTT, D. A. A further investigation of the chemical properties of insulin.....	601
RABINOWITCH, I. M. Urea tests of renal efficiency. I.....	617
HUNTER, GEORGE, AND EAGLES, BLYTHE A. The isolation from blood of a hitherto unknown substance, and its bearing on present methods for the estimation of uric acid. Plate 2.....	623
MAYNARD, L. A., GOLDBERG, S. A., AND MILLER, R. C. The influence of sunlight on bone development in swine.....	643
VICKERY, HUBERT BRADFORD. Some nitrogenous constituents of the juice of the alfalfa plant. VI. Asparagine and amino acids in alfalfa.....	657
SMITH, H. MONMOUTH, AND DOOLITTLE, DORTHA BAILEY. Energy expenditure of women during horizontal walking at different speeds.....	665
STERN, HANS T. The colorimetric pH test of water or unbuffered solutions.....	677
LEVENE, P. A. The mucoproteins of the snails, <i>Helix aspersa</i> and <i>Helix pomatia</i>	683
VAN SLYKE, DONALD D., HASTINGS, A. BAIRD, MURRAY, CECIL D., AND SENDROY, JULIUS, JR. Studies of gas and electrolyte equilibria in blood. VIII. The distribution of hydrogen, chloride, and bicarbonate ions in oxygenated and reduced blood.....	701
ATCHLEY, DANA W., AND NICHOLS, EMILY G. The influence of portein concentration on the conductivity of human serum.....	729
STADIE, WILLIAM C., AND ROSS, EFFIE C. A micro method for the determination of base in blood and serum and other biological materials.....	735
WILSON, D. WRIGHT, LONG, W. L., THOMPSON, H. C., AND THURLOW, SYLVA. Changes in the composition of the urine after muscular exercise.....	755
LILJESTRAND, S. H., AND WILSON, D. WRIGHT. The excretion of lactic acid in the urine after muscular exercise.....	773
HJORT, AXEL M. The influence of orally administered calcium salts on the serum calcium of normal and thyreoparathyroprivic dogs.....	783
Index to Volume LXV	797

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